

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date
22 April 2004 (22.04.2004)

PCT

(10) International Publication Number
WO 2004/033728 A2

(51) International Patent Classification⁷: C12Q 1/68

(21) International Application Number: PCT/NL2003/000690

(22) International Filing Date: 13 October 2003 (13.10.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/417,779 11 October 2002 (11.10.2002) US

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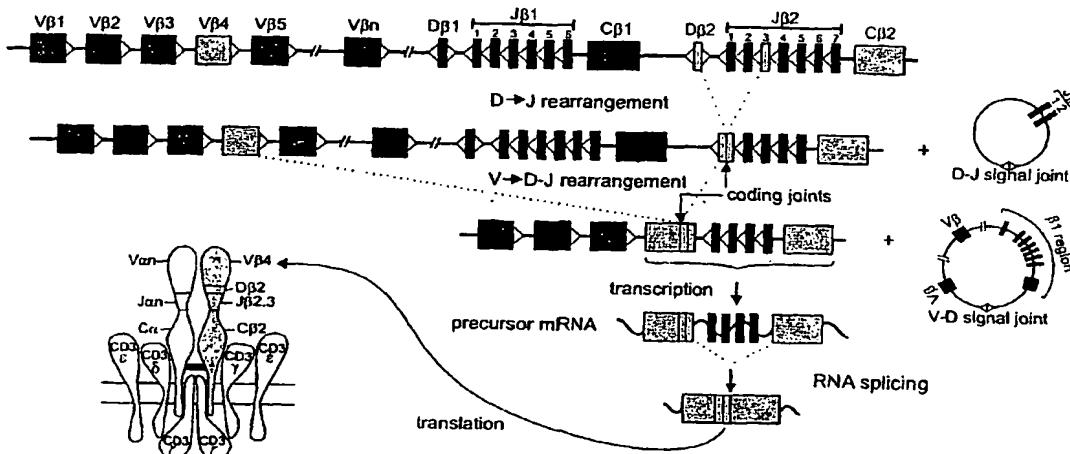
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(54) Title: NUCLEIC ACID AMPLIFICATION PRIMERS FOR PCR-BASED CLONALITY STUDIES



(57) Abstract: The invention relates to PCR-based clonality studies for among others early diagnosis of lymphoproliferative disorders. Provided is a set of nucleic acid amplification primers comprising a forward primer, or a variant thereof, and a reverse primer, or a variant thereof, capable of amplifying a rearrangement selected from the group consisting of a VH-JH IGH rearrangement, a DH-JH IGH rearrangement, a VK-JK IGK rearrangement, a VK/intron-Kde IGK rearrangement, a Vλ-Jλ IGL rearrangement, a VB-JB TCRB rearrangement, a Dδ-Jδ TCRD rearrangement, a Vγ-Jγ TCRG rearrangement, a Vδ-Jδ TCRD rearrangement, a Dδ-Dδ TCRD rearrangement, or a translocation selected from t(11;14)(BCL1-IGH) and t(14;18)(BCL2-IGH). The primers can be used in PCR-based clonality studies for early diagnosis of lymphoproliferative disorders and detection of minimal residual disease (MRD). Also provided is a kit comprising at least one set of primers of the invention.

WO 2004/033728 A2



(81) **Designated States (national):** AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, EG, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),

Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Title: Nucleic acid amplification primers for PCR-based clonality studies.

5 The present invention relates to PCR-based clonality studies for among others early diagnosis of lymphoproliferative disorders. In most patients with suspect lymphoproliferative disorders, histomorphology or cytomorphology supplemented with immunohistology or flow cytometric immunophenotyping can discriminate between malignant and reactive lymphoproliferations. However, in 5 to 10% of cases, making the
10 diagnosis is more complicated. The diagnosis of lymphoid malignancies can be supported by clonality assessment based on the fact that in principle all cells of a malignancy have a common clonal origin.

The majority of lymphoid malignancies belongs to the B-cell lineage (90 to 95%) and only a minority belongs to the T-cell lineage (5-7%) or NK-cell lineage (<2%). Acute
15 lymphoblastic leukemias (ALL) are of T-cell origin in 15 to 20% of cases, but in the group of mature lymphoid leukemias and in non-Hodgkin lymphomas (NHL) T-cell malignancies are relatively rare, except for specific subgroups such as cutaneous lymphomas (Table 1). Consequently, the vast majority of lymphoid malignancies (> 98%) contains identically (clonally) rearranged immunoglobulin (Ig) and/or T-cell receptor
20 (TCR) genes and in 25 to 30% of cases also well-defined chromosome aberrations are found, all of which can serve as markers for clonality.^{1,2}

The Ig and TCR gene loci contain many different variable (V), diversity (D), and joining (J) gene segments, which are subjected to rearrangement processes during early lymphoid differentiation.^{3,4} The V-D-J rearrangements are mediated via a recombinase
25 enzyme complex in which the RAG1 and RAG2 proteins play a key role by recognizing and cutting the DNA at the recombination signal sequences (RSS), which are located downstream of the V gene segments, at both sides of the D gene segments, and upstream of the J gene segments (Figure 1). Inappropriate RSS reduce or even completely prevent rearrangement.

30 The rearrangement process generally starts with a D to J rearrangement followed by a V to D-J rearrangement in case of Ig heavy chain (*IGH*), TCR beta (*TCRB*), and TCR delta (*TCRD*) genes (Figure 1) or concerns direct V to J rearrangements in case of Ig kappa (*IGK*), Ig lambda (*IGL*), TCR alpha (*TCRA*), and TCR gamma (*TCRG*) genes. The sequences between rearranging gene segments are generally deleted in the form of a

circular excision product, also called TCR excision circle (TREC) or B cell receptor excision circle (BREC) (Figure 1).

The Ig and TCR gene rearrangements during early lymphoid differentiation generally follow a hierarchical order. During B-cell differentiation: first the *IGH* genes rearrange, then *IGK*, potentially resulting in IgH/κ expression or followed by *IGK* deletion and *IGL* rearrangement, potentially followed by IgH/λ expression.⁵ This implies that virtually all Igλ⁺ B-cells have monoallelic or biallelic *IGK* gene deletions. During T-cell differentiation: first the *TCRD* genes rearrange, then *TCRG*, potentially resulting in TCRγδ expression or followed by further *TCRB* rearrangement and *TCRD* deletion with subsequent *TCRA* rearrangement, potentially followed by TCRαβ expression. The Ig and TCR gene rearrangement patterns in lymphoid malignancies generally fit with the above-described hierarchical order, although unusual rearrangement patterns are found as well, particularly in ALL.⁶

The many different combinations of V, D, and J gene segments represent the so-called combinatorial repertoire (Table 2), which is estimated to be ~2x10⁶ for Ig molecules, ~3x10⁶ for TCRαβ molecules and ~ 5x10³ for TCRγδ molecules. At the junction sites of the V, D, and J gene segments, deletion and random insertion of nucleotides occurs during the rearrangement process, resulting in highly diverse junctional regions, which significantly contribute to the total repertoire of Ig and TCR molecules, estimated to be >10¹².⁵

Mature B-lymphocytes further extend their Ig repertoire upon antigen recognition in follicle centers via *somatic hypermutation*, a process, leading to affinity maturation of the Ig molecules. The somatic hypermutation process focuses on the V-(D-)J exon of *IGH* and Ig light chain genes and concerns single nucleotide mutations and sometimes also insertions or deletions of nucleotides. Somatically-mutated Ig genes are also found in mature B-cell malignancies of follicular or post-follicular origin.⁷

Functionally rearranged Ig and TCR genes result in surface membrane expression of Ig, TCRαβ, or TCRγδ molecules. Based on the concept that only a single type of Ig or TCR molecule is expressed by a lymphocyte or lymphocyte clone, the clonally rearranged genes of mature lymphoid malignancies might be detectable at the protein level. Detection of single Ig light chain expression (Igκ or Igλ) has for a long time been used to discriminate between reactive (polyclonal) B-lymphocytes (normal Igκ/Igλ ratio: 0.7 – 2.8) versus aberrant (clonal) B-lymphocytes with Igκ/Igλ ratios of >4.0 or <0.5.⁸⁻¹⁰ In the vast majority (>90%) of mature B-cell malignancies, single Ig light chain expression can support the clonal origin of the malignancy.

Also, the development of many different antibodies against variable domains of the various TCR chains allows detection of monotypic V β , V γ and V δ domains, when compared with appropriate reference values.¹¹⁻¹⁶ In the interpretation of monotypic V β results using 20 to 25 antibodies against different V β families (Table 2), one should realize that clinically-benign clonal TCR $\alpha\beta^+$ T-cell expansions (frequently CD8 $+$) are regularly found in peripheral blood (PB) of older individuals.^{13, 17} These clonal T-cell expansions in PB are however relatively small in size: <40% of PB T-lymphocytes and <0.5x10⁶/ml PB.¹³ It is not yet clear to what extent such clinically benign T-cell clones can also be found in lymphoid tissues.

The results of monotypic V γ and V δ domain expression should be interpreted with caution, because in healthy individuals a large fraction of normal polyclonal TCR $\gamma\delta^+$ T-lymphocytes has been selected for V γ 9-J γ 1.2 and V δ 2-J δ 1 usage.^{18, 19} Consequently, high frequencies of V γ 9 $+$ /V δ 2 $+$ T-lymphocytes in PB should be regarded as a normal finding, unless the absolute counts are 1 to 2x10⁶/ml PB. It should be noted that most TCR $\gamma\delta^+$ T-cell malignancies express V δ 1 or another non-V δ 2 gene segment in combination with a single V γ domain (generally not V γ 9).^{15, 20}

Detection of Ig κ or Ig λ restricted expression or monotypic V β , V γ or V δ expression is relatively easy in flow cytometric studies of PB and bone marrow (BM) samples of patients with mature B-cell or T-cell leukemias. However, this appears to be more difficult in tissue samples with suspect lymphoproliferative disorders that are intermixed with normal (reactive) lymphocytes.

In contrast to the antibody-based techniques, molecular techniques are broadly applicable for detection of clonally rearranged Ig/TCR genes as well as well-defined chromosome aberrations. This previously concerned Southern blot analysis, but nowadays particularly PCR techniques are used.

Difficulties in making a final diagnosis of lymphoid malignancy occur in a proportion of cases (5 to 10%) despite extensive immunophenotyping. Therefore, additional (molecular clonality) diagnostics is needed to generate or to confirm the final diagnosis, such as in case of:

- any suspect B-cell proliferation where morphology and immunophenotyping are not conclusive;
- all suspect T-cell proliferations (CAUTION: T-cell rich B-NHL);
- lymphoproliferations in immunodeficient patients or transplanted patients;

patient or discrimination between a relapse and a second malignancy;

- further classification of a malignancy, e.g. via Ig/TCR gene rearrangement patterns or particular chromosome aberrations;
- 5 - occasionally: staging of lymphomas.

For long time, Southern blot analysis has been the gold standard technique for molecular clonality studies. Southern blotting is based on the detection of non-germline ("rearranged") DNA fragments, obtained after digestion with restriction enzymes. Well-chosen restriction enzymes (resulting in fragments of 2 to 15 kb) and well-positioned DNA probes (particularly downstream J segment probes) allow detection of virtually all Ig and TCR gene rearrangements as well as chromosome aberrations involving J gene segments.²¹⁻²⁸ It should be noted that Southern blot analysis focuses on the rearrangement diversity of Ig/TCR gene segments and therefore takes advantage of the 10 combinatorial repertoire.

15

Optimal Southern blot results for clonality assessment can particularly be obtained with the *IGH*, *IGK*, and *TCRB* genes, because these genes have an extensive combinatorial repertoire as well as a relatively simple gene structure which can be evaluated with only one or two DNA probes.^{22, 24, 28} The *IGL* and *TCRA* genes are more 20 complex and require multiple probe sets.^{25, 26, 29} Finally, the *TCRG* and *TCRD* genes have a limited combinatorial repertoire, which is less optimal for discrimination between monoclonality and polyclonality via Southern blot analysis.^{20, 21}

Despite the high reliability of Southern blot analysis, it is increasingly replaced by 25 PCR techniques, because of several inherent disadvantages: Southern blot analysis is time-consuming, technically demanding, requires 10 to 20 µg of high quality DNA, and has a limited sensitivity of 5 to 10%.²¹

Detection of rearranged Ig/TCR genes and chromosome aberrations by PCR techniques 30 requires precise knowledge of the rearranged gene segments in order to design appropriate primers at opposite sides of the junctional regions and breakpoint fusion regions, respectively.

In routine PCR-based clonality studies, the distance between the primers should be less than 1 kb, preferably less than 500 bp. This is particularly important for 35 discrimination between PCR products from monoclonal versus polyclonal Ig/TCR gene rearrangements, which is based on the diversity of the junctional regions (diversity in

size and composition). So far, mainly *IGH* and *TCRG* gene rearrangements have been used for PCR-based clonality studies, because of the limited number of primers needed to detect VH-JH and V γ -J γ rearrangements.

The main advantages of PCR techniques are their speed, the low amounts of DNA required, the possibility to use DNA of lower quality, and the relatively good sensitivity of 1 to 5%, for some types of rearrangements even <1%. Consequently, PCR techniques allow the use of small biopsies (e.g. fine needle aspiration biopsies), or the use of formaldehyde-fixed paraffin-embedded samples, which generally results in DNA of lower quality. Therefore also archival material might be used, if needed.

10

Molecular clonality studies can be highly informative, but several limitations and pitfalls might hamper the interpretation of the results obtained with conventional detection methods:

1. *Limited sensitivity, related to normal polyclonal background*

15 The detection limit varies between 1% and 10% (or even 15%), dependent on the applied technique (Southern blot analysis or PCR techniques) and dependent on the relative size of the "background" of normal (polyclonal) B- and T-lymphocytes. A limited sensitivity might hamper the detection of small clonal cell populations with less than 5 to 10% clonal lymphoid cells.

20 2. *Clonality is not equivalent to malignancy*

Detection of clonality does not always imply the presence of a malignancy. Some clinically benign proliferations have a clonal origin, such as many cases of CD8 $^{+}$ (or sometimes CD4 $^{+}$) T-lymphocytosis, benign monoclonal gammopathies, initial phases of EBV $^{+}$ lymphoproliferations (frequently being oligoclonal) in immunodeficient patients, 25 and benign cutaneous T-cell proliferations, such as lymphomatoid papulosis, etc. This implies that results of molecular clonality studies should always be interpreted in the context of the clinical, morphological, and immunophenotypic diagnosis, i.e. in close collaboration with hematologists, cytomorphologists, pathologists and immunologists.

30 3. *Ig and TCR gene rearrangements are not markers for lineage*

In contrast to the initial assumption, it is now clear for more than a decade that Ig and TCR gene rearrangements are not necessarily restricted to B-cell and T-cell lineages, respectively. Cross-lineage TCR gene rearrangements occur relatively frequently in immature B-cell malignancies, particularly in precursor-B-ALL (>90% of cases),³⁰ but also acute myeloid leukemias (AML) and mature B-cell malignancies might contain TCR 35 gene rearrangements.³¹⁻³³ Albeit at a lower frequency, also cross-lineage Ig gene

rearrangement occur in the immunoglobulin chain (*IGH*) locus.^{33,34}

Virtually all (>98%) $TCR\alpha\beta^+$ T-cell malignancies have *TCRG* gene rearrangements (generally biallelic) and many $TCR\gamma\delta^+$ T-cell malignancies have *TCRB* gene rearrangements, implying that the detection of *TCRB* or *TCRG* rearrangements is not indicative of T-cells of the $\alpha\beta$ or $\gamma\delta$ T-cell lineage, respectively, either.

In addition to these cross-lineage rearrangements, it has been established that several lymphoid malignancies have unusual Ig/TCR gene rearrangement patterns. This information is available in detail for precursor-B-ALL and T-ALL, but not yet for most other lymphoid malignancies.⁶

4. *Pseudoclonality and oligoclonality*

The detection of a seemingly clonal or seemingly oligoclonal lymphoid cell population (pseudoclonality) is rare in Southern blot analysis, unless genes with a limited combinatorial repertoire are used, such as *TCRG* or *TCRD*. This might result in faint rearranged bands, e.g. representing $V\gamma 9-J\gamma 1.2$ or $V\delta 2-J\delta 1$ rearrangements derived from antigen-selected $TCR\gamma\delta^+$ T-lymphocytes. Yet, this is a well-known pitfall of Southern blot analysis and will not result in rearranged bands of high density.

Pseudoclonality in PCR-based clonality studies is more difficult to recognize. The high sensitivity of PCR can cause amplification of the few Ig or TCR gene rearrangements derived from a limited number of B-cells or T-cells in the studied tissue sample. Particularly the few reactive (polyclonal) T-cells in a small needle biopsy or in a B-NHL sample with high tumor load might result in (oligo)clonal PCR products. Frequently the amount of such PCR products is limited. This is particularly seen when *TCRG* genes are used as PCR target. Duplicate or triplicate PCR analyses followed by mixing of the obtained PCR products should help to clarify whether the seemingly clonal PCR products are in fact derived from different lymphocytes.

Finally, reactive lymph nodes can show a reduced diversity of the Ig/TCR repertoire, caused by predominance of several antigen-selected subclones (oligoclonality). Particularly lymph nodes or blood samples of patients with an active EBV or CMV infection can show a restricted TCR repertoire or TCR gene oligoclonality. Also clinical pictures of immunosuppression are frequently associated with restricted TCR repertoires, e.g. in transplant patients or patients with hairy cell leukemia.³⁵ Recovery from transplantation and hematological remission are followed by restoration of the polyclonal TCR repertoire.^{36,37}

35 5. *False-positive results*

In Southern blot analysis, false-positive results are rare and can generally be prevented by checking for underdigestion and by excluding polymorphic restriction sites.²¹

False-positive PCR results comprise a serious problem, if no adequate analysis of the obtained PCR products is performed to discriminate between monoclonal or polyclonal PCR products. Such discrimination can be achieved via single-strand conformation polymorphism (SSCP) analysis,²² denaturing gradient gel electrophoresis (DGGE),²³ heteroduplex analysis (HD),^{40, 41} or GeneScanning (GS).^{42, 43} These techniques exploit the junctional region diversity for discrimination between monoclonal cells with identical junctional regions and polyclonal cells with highly diverse junctional regions.

6. *False-negative results*

False-negative results are rare in Southern blot analysis if appropriate J gene segment probes are used. Nevertheless, some uncommon rearrangements (generally non-functional rearrangements) might be missed, such as V-D rearrangements or deletions of the J regions. PCR analysis of Ig and TCR genes might be hampered by false-negative results because of improper annealing of the applied PCR primers to the rearranged gene segments. This improper primer annealing can be caused by two different phenomena. Firstly, precise detection of all different V, D, and J gene segments would require many different primers (Table 1), which is not feasible in practice. Consequently, family primers are designed, which specifically recognize most or all members of a particular V, D, or J family. Alternatively, consensus primers are used, which are assumed to recognize virtually all V and J gene segments of the locus under study. Family primers and particularly consensus primers are generally optimal for a part of the relevant gene segments, but show a lower homology (70 to 80%) to other gene segments. This may eventually lead to false-negative results, particularly in Ig/TCR genes with many different gene segments. In *TCRG* and *TCRD* genes this problem is minimal, because of their limited number of different gene segments.

The second phenomenon is the occurrence of somatic hypermutations in rearranged Ig genes of follicular and post-follicular B-cell malignancies, particularly B-cell malignancies with class-switched *IGH* genes.

Sufficient knowledge and experience can prevent the first four pitfalls, because they mainly concern interpretation problems. The last two pitfalls concern technical problems, which can be solved by choosing reliable techniques for PCR product analysis and by the design of better primer sets.

has resulted in the selection of reliable combinations of restriction enzymes (fragments between 2 and 15 kb, avoiding polymorphic restriction sites) and probes (mainly downstream of J gene segments). Although Southern blot analysis is a solid "gold standard" technique, many laboratories have gradually replaced Southern blot analysis by PCR technology, because PCR is fast, requires minimal amounts of medium-quality DNA, and has an overall good sensitivity.

Despite the obvious advantages, replacement of Southern blot analysis by PCR techniques for reliable Ig/TCR studies is hampered by two main technical problems:

- 10 - false negative results due to improper primer annealing;
- difficulties in discrimination between monoclonal and polyclonal Ig/TCR gene rearrangements.

Several individual diagnostic laboratories tried to solve the problems of the PCR-based 15 clonality studies, but thus far no reliably standardized PCR protocols were obtained. In contrast, many different primer sets are being used, which all differ in their sensitivity and applicability.

The present invention now provides sets of nucleic acid amplification primers and 20 standardized PCR protocols for detecting essentially all relevant Ig and TCR loci and two frequently occurring chromosome aberrations. The primers sets according to the invention comprising a forward and a reverse primer are capable of amplifying clonal rearrangements of the Ig heavy chain genes (*IGH*), Ig kappa chain genes (*IGK*), Ig lambda chain genes (*IGL*), TCR beta genes (*TCRB*), TCR gamma genes (*TCRG*), and TCR delta genes (*TCRD*) or of amplifying chromosomal translocation t(11;14)(*BCL1-IGH*) and 25 t(14;18)(*BCL2-IGH*). The primers of the invention allow that both complete and incomplete rearrangements are detectable and that gene segments from different V, (D), and J families can be recognized.

Two techniques which can be used in a method of the invention for 30 discrimination between monoclonal and polyclonal Ig/TCR gene rearrangements are heteroduplex analysis and GeneScanning. Heteroduplex analysis uses double-stranded PCR products and takes advantage of the length and composition of the junctional regions, whereas in GeneScanning single-stranded PCR products are separated in a high-resolution gel or polymer according to their length only (Figure 2).

107 different, specific primers for all the relevant Ig/TCR loci as well as for t(11;14) (*BCL1-IGH*) and t(14;18) (*BCL2-IGH*), or variants thereof, are provided (see Figures 3 to 11). The term "variant" refers to a primer which differs in 1 to 5 nucleotides, preferably 1 to 3 nucleotides, from the size and/or position from the nucleotide of a 5 primer sequence shown, provided that the nucleotide sequence of said variant primer contains at most 2 mismatches, at most 1 mismatch, most preferably no mismatches with the target locus. In addition, a variant primer comprises a (differentially) labeled primer, i.e. a primer having a label that can be identified or distinguished from other labels by any means, including the use of an analytical instrument. Examples of 10 differentially labeled primers are primers provided with a fluorescent label such as a 6-FAM, HEX, TET or NED dye. Labeled primers of the invention are particularly advantageous for use in automated high resolution PCR fragment analysis (Gene Scanning technology) for detection of PCR products. As is exemplified below, differentially labeled primers according to the invention allow to distinguish different 15 PCR amplification products of approximately the same length (size), preferably using multi-color GeneScanning. Of course, a variant nucleic acid amplification primer, be it a forward or a reverse (dye-labeled) primer, should not be capable of forming dimers with any other (variant) forward and/or reverse nucleic acid amplification primer that is used in an amplification reaction, since this can interfere with primer annealing to a target 20 locus and thus with the amplification of the rearrangement or translocation of interest.

In one embodiment, the invention provides a nucleic acid amplification assay, preferably a PCR assay, using at least one set of primers according to the invention. Said PCR assay can be a single (monoplex) or a multiplex PCR. In a preferred embodiment, a set of primers according to the invention is used in a standardized multiplex PCR assay, 25 using for example two or more forward primers, or three or four forward primers, or variants thereof (e.g. selected from a group of "family primers", for example from the V_H family primers), together with one or more consensus reverse primer(s), or variant(s) thereof (e.g. a J_H consensus primer). The family primers of the invention are designed in such a way that they recognize most or all gene segments of a particular family (see Table 30 2). In a specific embodiment, all 107 primers are used in only 18 multiplex PCR tubes: 5 for *IGH* (3x V_H - J_H and 2x D_H - J_H), 2 for *IGK*, 1 for *IGL*, 3 for *TCRB* (2x $V\beta$ - $J\beta$ and 1x $D\beta$ - $J\beta$), 2 for *TCRG*, 1 for *TCRD*, 3 for *BCL2-IGH*, and 1 for *BCL1-IGH* (Figures 3 to 11). Such an assay allows assessing clonal rearrangements and / or chromosome aberrations. Furthermore, it allows detection of a lymphoproliferative disorder. Multiplex PCR 35 testing of the primers on about 90 Southern blot defined lymphoproliferations showed

concordant.

In another embodiment, a method is provided for detecting a rearrangement, preferably two or more rearrangements, selected from the group consisting of a V_H - J_H 5 IGH rearrangement, a D_H - J_H IGH rearrangement, a V_K - J_K IGK rearrangement, a V_K /intron-Kde IGK rearrangement, a V_{λ} - J_{λ} IGL rearrangement, a V_{β} - J_{β} $TCRB$ rearrangement, a D_{δ} - J_{δ} $TCRB$ rearrangement, a V_{γ} - J_{γ} $TCRG$ rearrangement, a V_{δ} - J_{δ} $TCRD$ rearrangement, a D_{δ} - J_{δ} $TCRD$ rearrangement, a D_{δ} - D_{δ} $TCRD$ rearrangement, and a V_{δ} - D_{δ} $TCRD$ rearrangement, using at least one set of primers according to the 10 invention. Also provided is a method for detecting a $t(11;14)(BCL1-IGH)$ translocation or a $t(14;18)(BCL2-IGH)$ translocation, using at least one set of primers according to the invention. Furthermore, methods are provided for detecting at least one of the above rearrangements and at least one translocation, using at least two sets of primers as provided herein.

15 In a further aspect, a set of nucleic acid amplification primers capable of amplifying a human gene selected from the group consisting of the human $AF4$ gene (exon 3), the human $AF4$ gene (exon 11), the human $PLZF1$ gene, the human $RAG1$ gene and the human $TBXAS1$ gene is provided (see Fig. 12). Using one or more of these five 20 primer sets consisting of a forward primer (or a variant thereof) and a reverse primer (or a variant thereof) in a nucleic acid amplification assay of the invention, it is possible to detect one or more "Control Gene(s)" selected from the group consisting of the human $AF4$ gene (exon 3), the human $AF4$ gene (exon 11), the human $PLZF1$ gene, the human $RAG1$ gene and the human $TBXAS1$ gene. Such a detection method is advantageously 25 used to assess the quality (e.g. integrity and amplifiability) of a nucleic acid (DNA) sample extracted or isolated from a biological sample, for instance DNA extracted from a paraffin-embedded sample (see Example 10).

30 The ability of the different primer sets of the invention to amplify clonal rearrangements and/ or chromosomal aberrations (translocations) has been tested in many different types of malignant lymphomas, among which follicular lymphoma, diffuse large B-cell lymphoma, and multiple myeloma. It was found that a set of primers is very useful for assessing clonal rearrangements and/or chromosomal translocations. It appeared that the detection rate of clonal rearrangements using the multiplex primer tubes according to the invention is unprecedentedly high, i.e at least 95%.

35 Parallel testing of available paraffin-embedded tissues of the above samples revealed largely identical results, if the DNA quality of these tissues is sufficiently high,

meaning that fragments of at least 300 bp can be amplified in a specially-designed control gene PCR.

The applicability of the developed multiplex PCR assays according to the invention was evaluated on series of 50 to 100 cases per type of lymphoid malignancy.

5 Following national pathology panel review, and central pathology panel review in case of difficulties, all included cases were defined according to the World Health Organization (WHO) classification. The studied diagnostic categories included malignancies such as follicular lymphoma, mantle cell lymphoma, marginal zone lymphoma, diffuse large B-cell lymphoma, angioimmunoblastic T-cell lymphoma, peripheral T-cell lymphoma, and 10 anaplastic large cell lymphoma, as well as reactive lesions. The results show a very high level of clonality detection, even in entities, which are known to bear somatic hypermutations such as follicular lymphoma and diffuse large B-cell lymphoma. Particularly the usage of the three *IGH* V_H-J_H tubes, supplemented with the two *IGH* D_H-J_H tubes and the two *IGK* tubes appeared to be highly efficient in the detection of clonal Ig 15 gene rearrangements. This high efficiency is obtained by the complementarity of the Ig tubes as well as by the fact that D_H-J_H and *IGK*-Kde rearrangements are not (or rarely) somatically mutated. Such complementarity was also found for the *TCRB* and *TCRG* primers in case of T-cell malignancies.

20 Furthermore, interesting and unexpected rearrangement patterns, such as unusual cross-lineage rearrangements, were observed. Remarkably, in about 10% of reactive lesions clonal rearrangements were detected. These reactive lymphoproliferations included EBV-related lymphoproliferations and atypical hyperplasias like Castleman's disease, as well as lesions that were suspicious for a B- or T-cell clone.

25 In a specific embodiment, a method is provided for the detection of minimal residual disease (MRD). The term minimal residual disease (MRD) describes the situation in which, after chemotherapy for acute leukemia (AL), a morphologically normal bone marrow can still harbor a relevant amount of residual malignant cells. Detection of minimal residual disease (MRD) is a new practical tool for a more exact 30 measurement of remission induction during therapy because leukemic blasts can be detected down to 10⁻⁴–10⁻⁶. Known PCR-based MRD analysis uses clonal antigen receptor rearrangements detectable in ~90–95% of the investigated patient samples. However, amplification of polyclonal products often leads to false-positive PCR amplicons not suitable for MRD analysis. The invention now provides a method for the detection of 35 identically (clonally) rearranged Ig and TCR genes or detection of well-defined and

rearrangements and translocations detected using a set of primers of the invention not only serve as markers for clonality at diagnosis, but also as PCR targets for detection of MRD during follow-up.

5 In a further aspect, the invention provides a (diagnostic) kit for the detection of at least one rearrangement selected from the group consisting of a V_H - J_H IGH rearrangement, a D_H - J_H IGH rearrangement, a V_K - J_K IGK rearrangement, a V_K /intron-Kde IGK rearrangement, a V_λ - J_λ IGL rearrangement, a V_β - J_β $TCRB$ rearrangement, a D_β - J_β $TCRB$ rearrangement, a V_γ - J_γ $TCRG$ rearrangement, a V_δ - J_δ $TCRD$ rearrangement, a D_δ - J_δ $TCRD$ rearrangement, a D_δ - D_δ $TCRD$ rearrangement, a V_δ - D_δ $TCRD$ rearrangement and/or at least one translocation selected from $t(11;14)(BCL1-IGH)$ and $t(14;18)(BCL2-IGH)$, comprising at least one set of primers according to the invention. A kit of the invention is highly suitable for PCR-based clonality diagnostics. Optionally, such a kit also comprises at least one set of primers capable of amplifying a 10 human "control gene" as mentioned above. Inclusion of one, preferably at least two, more preferably at least three of these control gene primer sets in a Control Tube can be helpful in estimating the quality of the DNA sample to be diagnosed, for instance DNA extracted from paraffin-embedded tissue.

15

20 In a further aspect, the invention provides a method for rapid discrimination of different types of Ig/TCR gene rearrangements in the same multiplex PCR tube. GeneScanning allows the application of multiple different fluorochrome-conjugated primers in a single tube. Such differential labeling of primers can be used for extra discrimination between different types of Ig or TCR gene rearrangements.

25 Differential labeling of V primers generally has limited added value, but differential labeling of downstream primers can support the rapid and easy identification of the type of Ig/TCR gene rearrangement, which is useful for PCR-based detection of minimal residual disease.^{44,45} Labeling of J primers is not regarded to be informative for IGH (V_H - J_H or D_H - J_H), IGK (V_K - J_K), or IGL (V_λ - J_λ). For rapid identification of IGK -Kde rearrangements, it might be interesting to discriminate between V_K -Kde and intron 30 RSS-Kde rearrangements by differential labeling of the Kde and intron RSS primers (see Figure 5B).

35 The most informative multicolor GeneScanning can be designed for TCR gene rearrangements, facilitating the rapid recognition of the different types of $TCRB$, $TCRG$, and $TCRD$ gene rearrangements. For example, differential labeling of the $J\beta 1$ and $J\beta 2$ primers in $TCRB$ tube A (see Figure 7B) allows easy identification of the polyclonal and

monoclonal V β -J β 1 versus V β -J β 2 rearrangements (Figure 13A). Differential labeling of the J γ 1.3/2.3 and J γ 1.1/2.1 primers (Figure 8B) results in easy identification of the different types of *TCRG* gene rearrangements (Figure 13B). Differential labeling of the J δ primers, D δ 2 primer, and D δ 3 primer in the *TCRD* tube (Figure 9B) results in easy 5 identification of the most relevant *TCRD* gene rearrangements, such as D δ 2-J δ , V δ -J δ , D δ 2-D δ 3, and V δ 2-D δ 3 rearrangements (Figure 13C).

These multi-color multiplex PCR tubes appear to be easy and convenient in daily practise of PCR based clonality diagnostics.

Figure 1. Schematic diagram of sequential rearrangement steps, transcription, and translation of the *TCRB* gene. In this example first a D β 2 to J β 2.3 rearrangement occurs, followed by V β 4 to D β 2-J β 2.3 rearrangement, resulting in the formation of a V β 4-D β 2-J β 2.3 coding joint. The rearranged *TCRB* gene is transcribed into precursor mRNA, spliced into mature mRNA, and finally translated into a TCR β protein chain. The two extrachromosomal TCR excision circles (TRECs) that are formed during this recombination process are indicated as well; they contain the D-J signal joint and V-D signal joint, respectively.

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Figure 2. Schematic diagram of heteroduplex analysis and GeneScanning of PCR products, obtained from rearranged Ig and TCR genes. A. Rearranged Ig and TCR genes (*IGH* in the example) show heterogeneous junctional regions with respect to size and nucleotide composition. Germline nucleotides of V, D, and J gene segments are given in large capitals and randomly inserted nucleotides in small capitals. The junctional region heterogeneity is employed in heteroduplex analysis (size and composition) and GeneScanning (size only) to discriminate between products derived from monoclonal and polyclonal lymphoid cell populations. B. In heteroduplex analysis, PCR products are heat-denatured (5 min, 94°C) and subsequently rapidly cooled (1 hour, 20 4°C) to induce duplex (homo- or heteroduplex) formation. In cell samples consisting of clonal lymphoid cells, the PCR products of rearranged *IGH* genes give rise to homoduplexes after denaturation and renaturation, whereas in samples which contain polyclonal lymphoid cell populations the single-strand PCR fragments will mainly form heteroduplexes, which result in a background smear of slowly migrating fragments upon electrophoresis. C. In GeneScanning fluorochrome-labeled PCR products of rearranged *IGH* genes are denatured prior to high-resolution fragment analysis of the resulting single-stranded fragments. Monoclonal cell samples will give rise to PCR products of identical size (single peak), whereas in polyclonal samples many different *IGH* PCR products will be formed, which show a characteristic Gaussian size distribution.

30

Figure 3. PCR analysis of *IGH* (VH-JH) rearrangements. A. Schematic diagram of *IGH* gene complex on chromosome band 14q32.3 (adapted from ImMunoGeneTics database).^{46,47} Only rearrangeable non-polymorphic VH gene segments are included in blue (functional VH), or in gray (rearrangeable pseudogenes). Recently discovered (35 generally truncated) VH pseudogenes are not indicated. B. Schematic diagram of *IGH*

VH-JH rearrangement with three sets of VH primers and one JH consensus primer, combined in three multiplex tubes. The relative position of the VH and JH primers is given according to their most 5' nucleotide upstream (-) or downstream (+) of the involved RSS. The VH gene segment used as representative VH family member for primer design is indicated in parentheses. **C, D, and E.** Heteroduplex analysis and GeneScanning of the same polyclonal and monoclonal cell populations, showing the typical heteroduplex smears and homoduplex bands (left panels) and the typical polyclonal Gaussian curves and monoclonal peaks (right panels). The approximate distribution of the polyclonal Gaussian curves is indicated in nt.

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Figure 4. PCR analysis of *IGH* (DH-JH) rearrangements. **A.** Schematic diagram of *IGH* (DH-JH) rearrangement with seven DH family primers and one JH consensus primer, divided over two tubes (*IGH* tubes D and E). The DH7 (7-27) primer was separated from the other six DH primers, because the DH7 and JH consensus primer will give a germline PCR product of 211 nt. The relative position of the DH and JH primers is given according to their most 5' nucleotide upstream (-) or downstream (+) of the involved RSS. The DH gene segment used as representative DH family member for primer design is indicated in parentheses. **B and C.** Heteroduplex analysis (left panels) and GeneScanning (right panels) of the same polyclonal and monoclonal cell populations. The approximate distribution of the polyclonal and monoclonal peaks is indicated. The potential background band/peak in tube D is indicated with an asterisk and is located outside the expected range of DH-JH rearrangements. The germline DH7-JH band of tube E is also indicated with an asterisk.

25

Figure 5. PCR analysis of *IGK* gene rearrangements. **A.** Schematic diagram of the *IGK* gene complex on chromosome band 2p11.2 (adapted from ImMunoGeneTics database).^{46,47} Only rearrangeable non-polymorphic V_{κ} gene segments are indicated in blue (functional V_{κ}) or in grey (nonfunctional V_{κ}). The cluster of inverted V_{κ} gene segments (coded with the letter D) is located ~800 kb upstream of the non-inverted V_{κ} gene segments. These upstream V_{κ} gene segments are presented as a mirrored image to their corresponding non-inverted counterparts. **B.** Schematic diagrams of V_{κ} - J_{κ} rearrangement and the two types of Kde rearrangements (V_{κ} -Kde and intron RSS-Kde). The relative position of the V_{κ} , J_{κ} , Kde and intron RSS (INTR) primers is given according to their most 5' nucleotide upstream (-) or downstream (+) of the involved RSS. **35** The V_{κ} gene segment used as representative member of the $V_{\kappa}1$, $V_{\kappa}2$, and $V_{\kappa}3$ families

primers are divided over two tubes: tube A with $V\kappa$ and $J\kappa$ primers and tube B with $V\kappa$,
intron RSS, and Kde primers. **C and D.** Heteroduplex analysis and GeneScanning of the
same polyclonal and monoclonal cell populations, showing the typical heteroduplex
5 smears and homoduplex bands (left panels) and the typical Gaussian curves and
monoclonal peaks (right panels). The approximate distribution of the polyclonal
Gaussian curves is indicated in nt.

Figure 6. PCR analysis of *IGL* gene rearrangements. A. Schematic diagram of *IGL*
10 gene complex on chromosome band 22q11.2 (adapted from ImMunoGenetics database).⁴⁸

⁴⁹ Only rearrangeable non-polymorphic $V\lambda$ gene segments are included in blue (functional
 $V\lambda$) or in grey (nonfunctional $V\lambda$) **B.** Schematic diagram of $V\lambda$ - $J\lambda$ rearrangement with
two $V\lambda$ family primers and one $J\lambda$ consensus primer. Only two $V\lambda$ primers were designed
15 for $V\lambda 1$ plus $V\lambda 2$ and for $V\lambda 3$, because these three $V\lambda$ families cover approximately 70%
of rearrangeable $V\lambda$ gene segments and because approximately 90% of all *IGL* gene
rearrangements involve $V\lambda 1$, $V\lambda 2$, or $V\lambda 3$ gene segments. ⁴⁹ Although five of the seven $J\lambda$
gene segments can rearrange, only a single $J\lambda$ consensus primer was designed for $J\lambda 1$,
 $J\lambda 2$, and $J\lambda 3$, because 98% of all *IGL* gene rearrangements involve one of these three
gene segments. ⁴⁹ The relative position of the $V\lambda$ and $J\lambda$ primers is given according to
20 their most 5' nucleotide upstream (-) or downstream (+) of the involved RSS. **C.**
Heteroduplex analysis and GeneScanning of the same polyclonal and monoclonal cell
populations, showing the typical heteroduplex smears and homoduplex bands (left panel)
and the polyclonal Gaussian curves and monoclonal peaks (right panel). The
approximate position of the polyclonal Gaussian curves is indicated in nt.

Figure 7. PCR analysis of *TCRB* gene rearrangements. A. Schematic diagram of
the human *TCRB* locus. The gene segment designation is according to Arden et al.⁵⁰ with
the designation according to Rowen et al.⁵¹ and Lefranc et al.^{46,47} in parentheses. The
figure is adapted from the international ImMunoGeneTics database.^{46,47} Only the
30 rearrangeable non-polymorphic $V\beta$ gene segments are depicted in blue (functional $V\beta$), in
half blue/half gray (potential functional, but no protein expression found) and in grey
(non-functional $V\beta$). **B.** Schematic diagram of $V\beta$ - $J\beta$ and $D\beta$ - $J\beta$ rearrangements. The 23
 $V\beta$ primers, 13 $J\beta$ primers and two $D\beta$ primers are combined in three tubes: tube A with
23 $V\beta$ primers and nine $J\beta$ primers, tube B with 23 $V\beta$ primers and four $J\beta$ primers, and
35 tube C with two $D\beta$ primers and 13 $J\beta$ primers. The 23 $V\beta$ primers and the 13 $J\beta$

primers are aligned in order to obtain comparably sized PCR products (see panels C and D). The V β primers cover approximately 90% of all V β gene segments. The relative position of the V β , D β , and J β primers is given according to their most 5' nucleotide upstream (-) or downstream (+) of the involved RSS. **C, D, and E.** Heteroduplex analysis

5 and GeneScanning of the same polyclonal and monoclonal cell populations, showing the typical heteroduplex smears and homoduplex bands (left panels) and the typical polyclonal Gaussian curves and monoclonal peaks (right panels). The approximate distribution of the polyclonal Gaussian curves is indicated in nt.

10 **Figure 8. PCR analysis of TCRG gene rearrangements.** **A.** Schematic diagram of the human TCRG locus on chromosome band 7p14. Only the rearrangeable V γ gene segments are depicted in blue (functional V γ) or in gray (non-functional V γ). For the J γ gene segments, both nomenclatures are used.^{46,47,52} **B.** Schematic diagram of TCRG V γ -J γ rearrangement with four V γ primers and two J γ primers, which are divided over two tubes. The relative position of the V γ and J γ primers is indicated according to their most 5' nucleotide upstream (-) or downstream (+) of the involved RSS. **C and D.** Heteroduplex analysis and GeneScanning of the same polyclonal and monoclonal cell populations, showing the typical heteroduplex smears and homoduplex bands (left panels) and the typical polyclonal Gaussian curves and monoclonal peaks (right panels). The approximate distribution of the polyclonal Gaussian curves is indicated in nt.

15 **Figure 9. PCR analysis of TCRD gene rearrangements.** **A.** Schematic diagram of human TCRD locus on chromosome band 14q11.2. The six "classical" V δ gene segments are indicated in blue, scattered between the V α gene segments in black. Since V δ 4, V δ 5, and V δ 6 are also recognized as V α gene segments, their V α gene code is given in parenthesis. **B.** Schematic diagram of V δ -J δ , D δ 2-J δ , D δ 2-D δ 3, and V δ -D δ 3 rearrangements, showing the positioning of six V δ , four J δ , and two D δ primers, all combined in a single tube. The relative position of the V δ , D δ , and J δ primers is indicated according to their most 5' nucleotide upstream (-) or downstream (+) of the involved RSS. **C.** Heteroduplex analysis (left panel) and GeneScanning (right panel) of the same polyclonal and monoclonal cell populations. The polyclonal cell populations show a vague smear in heteroduplex analysis and a complex and broad peak pattern in GeneScanning. The monoclonal bands and peaks are clearly visible. The approximate position of the PCR products of the different types of rearrangements in GeneScanning is indicated.

Figure 10. Detection of *BCL1*-IGH rearrangements. **A.** Schematic diagram of the *CCND1* gene and the *BCL1* breakpoint region MTC on chromosome band 11q13 as well as the JH gene segment on chromosome band 14q32. For the primer design in the *BCL1*-MTC region an artificial *BCL1*-MTC/JH4 junctional sequence was composed (as partially reported for JVM2⁵³): the first 50-nucleotides as reported by Williams⁵⁴ were linked to nucleotide 1-439 from MTC-sequence present at NCBI (accession-number S77049⁵⁵); the N-region "GCCG" of JVM2⁵³ was added followed by nucleotide 1921-3182 representing the JH4-JH6 genomic region (accession-number J00256). **B.** Agarose gel electrophoresis of a series of *BCL1*-IGH PCR products from different MCL patients and the positive control cell line JVM2. The PCR products differ in size, indicating different positions of the *BCL1*-MTC breakpoints. The larger bands of lower density represent PCR products that extend to the next downstream germline JH gene segment.

Figure 11. PCR detection of *BCL2*-IGH rearrangements. **A.** Schematic diagram of the *BCL2* gene on chromosome band 18q21. The majority of the *BCL2* breakpoints cluster in three regions: MBR, 3' MBR, and mcr. Consequently, multiplex primers have been designed to cover the potential breakpoints in these three regions: two MBR primers, four 3' MBR primers, and three mcr primers. The relative position of the *BCL2* primers is indicated according to their most 5' nucleotide upstream (-) or downstream (+) to the 3' end of *BCL2* exon 3 (according to NCBI accession no. AF325194S1), except for two *BCL2*-mcr primers; their position is indicated downstream of the first nucleotide of the AF275873 sequence. **B, C, and D.** Agarose gel electrophoresis of PCR products from different FCL patients and several positive control cell lines (DoHH2, K231, OZ, and SC1). Panel B and D contain the same samples and show complementarity in positivity, illustrating that tube C (mcr tube) has added value. The PCR products differ in size, related to different position of the *BCL2* breakpoints. The larger bands of lower density in the same lanes represent PCR products that extend to the next downstream germline JH gene segment or to the next upstream *BCL2* primer.

Figure 12. Control gene PCR for assessment of amplifiability and integrity of DNA samples. **A.** Schematic diagram of five control genes exons and the five primer sets for obtaining PCR products of 600 bp, 400 bp, 300 bp, 200 bp, and 100 bp. The relative position of the control gene primers is given according to their most 5' nucleotide downstream of the 5' splice site of the involved control gene exon. **B.** Control gene PCR

products of six DNA samples, separated in a 6% polyacrylamide gel. Two control samples contained high molecular weight DNA (outer lanes) and four DNA samples were obtained from paraffin-embedded tissue samples, showing reduced amplifiability (e.g. GBS-4 50 ng versus GBS-4 500 ng) or reduced integrity of the DNA (PT-4).

5

Figure 13. Multicolor GeneScanning for supporting the rapid and easy identification of TCR gene rearrangements. A. Two-color analysis of *TCRB* tube A with differential labeling of $J\beta 1$ primers (TET-labeled; green) and $J\beta 2$ primers (FAM-labeled; blue). The top panel nicely shows the two polyclonal $J\beta 1$ and $J\beta 2$ rearrangement patterns (c.f. Figure 7C), whereas the other two panels show clonal $J\beta 2$ rearrangements. B. Two-color analysis of *TCRG* tube A with differential labeling of the $J\gamma 1.3/2.3$ primer (FAM-labeled; blue) and the $J\gamma 1.1/2.1$ (TET-labeled; green). The top panel nicely shows the polyclonal rearrangement patterns (c.f. Figure 8C), whereas the other two panels show clonal $J\gamma 1.3/2.3$ and clonal $J\gamma 1.1/2.1$ rearrangements, respectively. C. Three-color analysis of *TCRD* gene rearrangements with differential labeling of $J\delta$ primers (FAM-labeled; blue), $D\delta 2$ primer (HEX-labeled; green) and $D\delta 3$ primer (NED-labeled; black). Within the complex rearrangement patterns of the *TCRD* tube (Figure 9C), the three-color analysis allows direct detection of $V\delta$ - $J\delta$ rearrangements (blue peaks), $D\delta 2$ - $J\delta$ rearrangements (blue and green peaks, not fully comigrating because of differences in migration speed of the two fluochromosomes), $V\delta 2$ - $D\delta 3$ rearrangement (black peaks), and $D\delta 2$ - $D\delta 3$ rearrangement (comigrating green and black peaks).

MATERIALS AND METHODS

Selection of PCR targets: aiming for complementarity

It was decided to aim for the availability of at least one PCR-detectable clonality target in each lymphoid malignancy. In mature B-cell malignancies this aim might be hampered by the occurrence of somatic hypermutations in Ig genes, which are particularly found in follicular and post-follicular B-cell malignancies. Therefore it was decided to include PCR targets that have some degree of complementarity.

Several rationales were used for target selection:

- *IGH* genes: not only complete V_H - J_H rearrangements but also incomplete D_H - J_H rearrangements were included as PCR targets, because D_H - J_H rearrangements are probably not affected by somatic hypermutations;
- *IGK* and *IGL* genes: both Ig light chain genes were included as PCR targets, because this increases the chance of finding a PCR-detectable Ig gene rearrangement in each mature B-cell malignancy;
- *IGK* genes: not only V_K - J_K rearrangements were included, but also rearrangements of the kappa deleting element (Kde), because they occur on one or both alleles in (virtually) all $Ig\lambda^+$ B-cell malignancies and in one third of $Ig\kappa^+$ B-cell malignancies and because Kde rearrangements are probably not affected by somatic hypermutation;
- *TCRB* genes: both complete $V\beta$ - $J\beta$ and incomplete $D\beta$ - $J\beta$ rearrangements, because complete and incomplete *TCRB* gene rearrangements occur in all mature $TCR\alpha\beta^+$ T-cell malignancies and also in many $TCR\gamma\delta^+$ T-cell malignancies;
- *TCRG* genes: this classical PCR clonality target is useful in all T-cell malignancies of the $TCR\gamma\delta$ and the $TCR\alpha\beta$ lineage.
- *TCRD* genes: this is a potentially useful target in immature T-cell malignancies as well as in $TCR\gamma\delta^+$ T-cell malignancies;
- *TCRA* gene: this gene was not included as PCR target, because of its high degree of complexity with ~50 V and 61 J gene segments. Furthermore, all T-cell malignancies with *TCRA* gene rearrangements contain *TCRB* gene rearrangements and generally also have *TCRG* gene rearrangements;
- functional gene segments: most suspect lymphoproliferations concern mature lymphocytes, which consequently have functional Ig or TCR gene rearrangements.

Therefore PCR primer design aimed at inclusion of (virtually) all functional Ig/TCR gene segments.

5 - well-defined chromosome aberrations: t(11;14) with *BCL1-IGH* and t(14;18) with *BCL2-IGH* were included as additional targets, because these two aberrations are PCR-detectable at relatively high frequencies in lymphomas i.e. in 30% of mantle cell lymphoma (MCL) and in 60 to 70% of follicular cell lymphomas (FCL), respectively.

Primer design for multiplex PCR

10 Precise detection of all V, D, and J gene segments in rearranged Ig and TCR genes would require many different primers (Table 2). For some gene complexes this might be possible (e.g. *TCRG* and *TCRD*), but for other loci in practice this is impossible because of the high number of different gene segments. To solve this problem, family primers can be designed, which recognize most or all gene segments of a particular family (Table 2). Alternatively, consensus primers can be made, which recognize conserved sequences that 15 occur in many or all involved gene segments.

20 The design of family primers and consensus primers balances between a limited number of primers and maximal homology with all relevant gene segments. In this study, we aimed at maximal homology with all relevant gene segments (particularly functional gene segments) in order to prevent suboptimal primer annealing, which might cause false-negative results. Furthermore, we aimed at the design of specific family primers without cross-annealing to other families

25 In order to limit the number of PCR tubes per locus, multiplexing of PCR primers became important for practical reasons. Consequently, special guidelines were developed to ensure maximal possibilities for designing primers useful in multiplex PCR tubes. For this purpose dr. W. Rychlick (Molecular Biology Insights, Cascade, CO, USA) provided his specially-adapted OLIGO 6.2 software program and supported the development of the guidelines for optimal primer design.

30 The general guidelines for primer design were as follows:

- the position of the primers should be chosen in such a way that the size of the PCR products would preferably be <300 bp (preferably 100 to 300 bp) in order to be able to use paraffin-embedded material;

- a minimal distance to the junctional region of preferably >10-15 bp should be taken into account (in order to avoid false-negativity due to impossibility of the 3' end of the

germline sequence);

- primers preferably should not be too long (e.g. <25 nucleotides).

5 The following parameters were used for primer design with the OLIGO 6.2 program:

- search for primers should be performed with *moderate* stringency;
- primer efficiency (PE) value should preferably be ~400 (and >630, if the primer is to be used as consensus primer for other gene segments as well);
- the most stable 3' dimer of upper/upper, lower/lower, or upper/lower primers should not exceed -4 Kcal (moderate search strategy); the most stable dimer overall being less important;
- in view of multiplex PCR, the following guidelines were taken into account: a common primer would have to be designed in the most consensus region (i.e. high PE in consensus search), whereas individual primers (family or member) have to be designed in the least consensus region (i.e. low PE value of that primer for gene segments that should not be covered) to avoid cross-annealing to other gene segments and thereby multiple (unwanted) PCR products.

PCR protocol

20 A standardised PCR protocol was developed based on pre-existing experience from earlier European collaborative studies. After initial testing and approval, the protocol was accepted as summarized in Table 3.

Techniques for analysis of PCR products obtained from Ig/ TCR gene rearrangements

The PCR products obtained from Ig and TCR gene rearrangements have to be analysed for discrimination between monoclonal lymphoid cells with identical junctional regions and polyclonal lymphoid cells with highly diverse junctional regions.

30 Based on the combined experience of the participating laboratories, two techniques were selected: heteroduplex (HD) analysis and Gene Scanning (GS) analysis. HD analysis uses double-stranded PCR products and takes advantage of the length and composition of the junctional regions, whereas in GS single-stranded PCR products are separated in a high resolution gel or polymer according to their length only (Figure 2).

Heteroduplex analysis of PCR products

PCR products obtained with *unlabeled* primers are denatured at high temperature (~95°C for 5 min), followed by rapid random renaturation at low temperature (preferably 5 at 4°C for 1 hour). This enforced duplex formation results in many different heteroduplexes with different migration speed in case of polyclonal lymphoproliferations, but resulting in homoduplexes with identical rapid migration in case of monoclonal lymphoproliferations. Electrophoresis of the homoduplexes in a 6% polyacrylamide gel results in a single band of predictable size, whereas the heteroduplexes form a smear at 10 a higher position (Figure 2). The heteroduplex technique is rapid, simple and cheap (see Table 4 for technical details) and has a detection limit of ~ 5 %.^{40,41} The detection limit is influenced by the frequency of polyclonal lymphocytes, because the formation of many heteroduplexes will also consume a part of the monoclonal PCR products.⁴¹

Genescanning analysis of PCR products

The PCR primers for GeneScanning analysis need to be labeled with a fluorochrome to allow detection of the PCR products with automated sequencing equipment (Figure 2).

22 The fluorochrome labeled single-strand (denatured) PCR products are size-separated in a denaturing polyacrylamide sequencing gel or capillary sequencing 20 polymer and detected via automated scanning with a laser (see Table 5 for technical details). This results in a Gaussian distribution of multiple peaks, representing many different PCR products in case of polyclonal lymphoproliferations, but gives a single peak consisting of one type of PCR product in case of a fully monoclonal lymphoproliferation 25 (Figure 2).

25 GeneScanning is rapid and relatively simple, but needs expensive equipment. GeneScanning is generally more sensitive than heteroduplex analysis and can reach sensitivities of 0.5 to 1% of clonal lymphoid cells.

Control genes and paraffin-embedded tissues

30 In several European countries, fresh tissue material is not easily available for molecular diagnostics such as PCR-based clonality studies. Therefore one of the aims of the present study was to develop a strategy for PCR-based clonality studies in paraffin-embedded tissues.

35 To control for the quality and amplifiability of DNA from paraffin-embedded material, a special multiplex control gene PCR was developed, resulting in a ladder of

described 90 cases sufficient paraffin-embedded tissue was available for DNA extraction. These DNA samples were tested in parallel to the freshly-obtained DNA samples, using the Control Gene multiplex tube as well as the Ig/TCR/*BCL1/BCL2* multiplex tubes for 5 clonality diagnostics (see Example 10).

EXAMPLE 1. Complete *IGH* gene rearrangements: V_H-J_H

Background

The functional rearrangement of the *IGH* gene, first D_H to J_H and subsequently V to D_H-J_H, is followed by antibody expression, the hallmark of mature B-cells. The *IGH* gene is located on chromosome 14q32.3 in an area covering approximately 1250 kilobases. 46 to 52 functional VH segments (depending on the individual haplotype) have been identified, which can be grouped according to their homology in six or seven VH subgroups. In addition approximately 30 non-functional VH segments have been described. Furthermore, 27 DH segments and functional six JH segments have been 10 15 consistently found (Table 2 and Figure 3A).⁵⁶

The VH segments contain three framework (FR) and two complementarity determining regions (CDR) (Figure 3B). The FRs are characterized by their similarity among the various VH segments whereas the CDRs are highly different even within the same VH family. Furthermore, the CDRs represent the preferred target sequences for 20 somatic hypermutations in the course of the germinal center reaction, which increase the variability within those regions. Although the FRs are usually less affected by somatic mutations, nucleotide substitutions may also occur within these regions, especially in B-cells under a heavy mutational process.

The highly variable V-D-JH regions can be amplified by PCR to detect clonal B-cell populations indicative of the presence of a malignant B-cell disorder. Clonal B-cells 25 can be discriminated from polyclonal B-cells (i.e. normal or reactive lymphoid tissues) based on the identical size and composition of the clonal PCR products as compared to the many different polyclonal PCR products with a size range of approximately 60 bp, 30 arranged in a Gaussian distribution. PCR-based strategies for detection of clonal B-cell populations in histological sections and cell suspensions have already been established in the early nineties. However, the initial PCR protocols used single VH consensus primers which were able to bind to one of the three framework regions, mainly FR3. Such consensus primers were not suitable to amplify all VH segments with the same 35 efficiency leading to non-detectability of a significant number of clonal rearrangements.

In addition, somatic mutations introduced in the course of the germinal center reaction are not restricted to the CDRs, but can also occur in FRs, thereby preventing primer annealing and consequently leading to absence of clonal PCR products despite the presence of a neoplastic B-cell population. This is especially true for follicular

5 lymphomas, diffuse large B-cell lymphomas, and multiple myelomas which usually contain high numbers of somatic mutations.

To further increase the detection rate of the *IGH* PCR, several attempts have been made to design family-specific primers to overcome the limitations of consensus primers. However, these family-specific primers are largely based on the sequences of

10 the previous consensus primers. Although these PCR strategies have helped to improve the detection rate, there is still a need of primer systems which are less sensitive to somatic hypermutations, thus allowing amplification of (virtually) all possible V-D-JH rearrangements.

15 Primer design

According to the guidelines of the invention, three sets of VH primers were designed with the help of the OLIGO-6.2 program corresponding to the three VH framework regions (FR1, FR2 and FR3) (Figure 3B). Each set of primers consisted of six or seven oligonucleotides capable to anneal to their corresponding VH segments (VH₁ to

20 VH₇) with no mismatches for most VH segments and one or at most two mismatches for some rare VH segments. The design was such that mismatches would be located at the very 5'-end of the primer. These VH primer sets were used in conjunction with a single JH consensus primer, designed to anneal to the most homologous 3'-end of the six JH segments, approximately 35 bp downstream of the JH RSS. This ensures that all JH

25 segments are detectable with the same binding efficiency and that the primer binding will not easily be affected by extensive nucleotide deletion in the course of the rearrangement process. In addition, there was no cross-annealing between the VH primers and the JH primer as evaluated by the OLIGO-6.2 program.

The JH primer was also designed to be used for amplification of other PCR

30 targets, such as incomplete DH-JH rearrangements as well as t(11;14) (*BCL1-IGH*) and t(14;18) (*BCL2-IGH*). This allows the detection of different PCR products by GS analysis employing the same labeled JH primer.

The initial testing of the newly designed VH-JH PCR was done by separate application of each VH primer together with the JH primer in an individual PCR. For this purpose, DNA extracted from B-cell lines as well as well-defined clonal patient samples was used. Furthermore, clonal rearrangements were tested for sensitivity by serial dilution in DNA extracted from reactive tonsils. Clonal control samples were not available for each possible *IGH* rearrangement, but all major VH segments and several rarely rearranged VH segments have been included in the initial testing phase.

All primer pairs worked with high efficiency and sensitivity. The expected clonal VH rearrangements were detectable and the sensitivity was at least 1% (10^{-2}). There was no background within the expected size range and the amplification of tonsillar DNA gave the expected Gaussian distribution curve. (Figure 3C, D, and E)

Based on these results we started the next phase of the initial primer testing by combining the VH primers into three sets, each specific for one of the three framework regions, which were used together with the common JH primer (Figure 3B). The results were the same as those obtained with single primer pairs, but with a slightly lower sensitivity. In addition, no nonspecific products were amplified within the expected size range, with the exception of a 340 bp PCR product which appeared in the FR1 multiplex PCR. This PCR product was generated irrespective of the source of the DNA (lymphoid and non-lymphoid) used for PCR, whereas no PCR product was obtained when no DNA template was applied. Furthermore, this amplicon was only detectable in heteroduplex analysis, not in GeneScanning. This indicates that the fluorescent labeled JH primer was not involved in the generation of this PCR product. Sequence analysis of this PCR product disclosed a VH4 fragment amplified by the FR1 VH4 primer in conjunction with the FR1 VH2 primer which apparently acted as a downstream primer by binding to the intronic VH4 sequence. This problem could be solved by designing a new FR1 VH2 primer which was located 25 bp upstream to the previous primer binding site.

Results of general testing phase

The approved *IGH* PCR was applied to the 90 Southern blot defined DNA samples, which were derived from well-characterized cases. Six of the 11 laboratories involved in the general testing phase performed GS analysis of the PCR products and five performed HD analysis. In addition several polyclonal as well as monoclonal samples (cell line DNA) were included as controls. 45 of these cases displayed dominant PCR products after GS analysis and 40 cases after HD detection, indicating the presence of a

monoclonal B-cell population. The clonal rearrangements were detectable with all three FR primer sets in 33 of the 45 clonal cases (GS) and in the remaining 12 with one or two of the three FR primer sets. It was concluded that most negative results were caused by somatic hypermutations in the primer binding site, preventing primer annealing and thus amplification.

The comparison of the VH-JH PCR results with the Southern blot results revealed a high degree of concordance. 85% (46 out of 55) and 76% (42 out of 55) of the samples with rearranged VH genes by Southern blot analysis showed a dominant amplification product by GS analysis and HD analysis, respectively. Vice versa, all but two samples harboring germline VH genes by Southern blot displayed a polyclonal pattern by GS and HD analysis.

Conclusion

In conclusion, the three multiplex PCRs for detection of clonal VH-JH rearrangements provide a new and reliable assay to identify clonal B-cell proliferations. The combined use of standardized primers in the three different FRs helps to decrease the rate of false-negative results due to somatic hypermutation in primer binding sites of the involved VH gene segments. **EXAMPLE 2. Incomplete *IGH* gene rearrangements: DH-JH**

Background

The formation of complete V-D-J rearrangements in the *IGH* locus on chromosome 14q32.3 is a sequential process that occurs in two steps: VH coupling is generally preceded by an initial rearrangement between DH and JH gene segments in early precursor-B cells (reviewed by ⁵⁷). In addition to the many distinct VH gene segments and the six functional JH gene segments (see Example 1), the human *IGH* locus also contains 27 DH gene segments.⁵⁸ Based on sequence homology, the 27 DH segments can be grouped into seven families: DH1 (formerly known as DM), DH2 (DLR), DH3 (DXP), DH4 (DA), DH5 (DK), DH6 (DN), and DH7 (DQ52); all families comprise at least four members, except for the seventh which consists of the single DH7-27 segment just upstream of the JH region (Figure 3A).^{58,59}

Recombination between any of the DH and JH segments will result in the formation of incomplete DH-JH joints, which can easily be detected in bone marrow-derived CD10⁺ / CD19⁻ precursor B-cells^{60,61} and hence also in a subset (20-25 %) of precursor B-cell acute lymphoblastic leukemias, which show an immature genotype.⁶² Sequencing revealed a predominance of DH2 (DH2-2), DH3 (DH3-9), and DH7-27 gene

respectively.⁶²

However, also in mature B-cell malignancies incomplete DH-JH rearrangements have been reported.^{61,63} Moreover, even in a subset of IgH-negative multiple myelomas, which can be considered as the most mature type of B-lineage malignancy, DH-JH joints were observed.⁶⁴ These DH-JH rearrangements were derived from the non-coding second allele and involved segments from DH1 to DH4 families.⁶⁴ Based on the description of DH-JH joints in precursor-B-ALL and multiple myelomas, it is assumed that incomplete DH-JH rearrangements are also present in other types of B-cell leukemias and lymphomas.

In immature T-cell malignancies DH-JH couplings have been identified as cross-lineage rearrangements;³⁴ interestingly, these almost exclusively occurred in the more immature non-TCR $\alpha\beta^+$ T-ALL subset and mainly involved the more downstream DH6-19 and DH7-27 segments. The latter segment is frequently (up to 40%) used in fetal B cells but rarely in adult B cells.^{65,66} Human adult precursor and mature B cells mainly seem to use DH2 and DH3 family segments, as evidenced from sequences of complete VH-DH-JH rearrangements.⁶⁶

Although the exact frequencies of incomplete DH-JH couplings in different types of mature B-cell malignancies are largely unknown, it is clear that they will at least be lower than those of VH-JH joinings. Nevertheless, DH-JH rearrangements might still represent an important complementary target for PCR-based clonality assessment. This presumed contribution of DH-JH rearrangements as PCR target is based on the assumption that incomplete rearrangements in the *IGH* locus will not contain somatic hypermutations, because transcription starting from the promoters in the V gene segments does not occur, which is regarded as an essential prerequisite for somatic hypermutation to take place.^{67,68} Especially in those types of B-lineage proliferations in which somatic hypermutations are frequent, PCR analysis of a possible DH-JH recombination product might therefore be relevant, and sometimes even the only possibility to detect the B-cell clone.

30 Primer design

Based on the high degree of homology within each DH family, seven family-specific DH primers were designed (Figure 4) in combination with the consensus JH primer that is also used for detection of VH-JH rearrangements (see Example 1) and t(11;14) (*BCL1-IGH*) and t(14;18) (*BCL2-IGH*) (Examples 8 and 9). Primers were designed such that cross-annealing to other DH family segments would be minimal or

preferably absent, resulting in distinct positions for the various family primers relative to the RSS elements (Figure 4). The expected PCR product sizes of DH-JH joints range from 110-130 bp (for DH7-JH joinings) to 395-415 bp (for DH3-JH rearrangements). Of note, due to the position of the DH7-27 segment close to the segments in the JH region, 5 PCR products of 211 bp (and also 419,1031,1404,1804, and 2420 bp in case of primer annealing to downstream JH gene segments) will be amplified from non-rearranged alleles and will be detected as a ladder of germline bands in virtually every sample.

Results of initial testing phase

10 For initial testing of the individual DH primers, high tumor load precursor B-ALL or T-ALL samples with well-defined clonal DH-JH rearrangements were used. Under standard PCR conditions using 1.5 mM MgCl₂ and AmpliTaq Gold buffer, all seven primer combinations appeared to detect the clonal DH-JH targets with product lengths within the expected size ranges. Cross-annealing of the DH primers to rearranged gene 15 segments from other DH families was only very weak or not observed at all. Furthermore, also in healthy control tonsillar or MNC DNA PCR products of the correct size ranges were observed. Nonspecific annealing of the primers was not observed for virtually all primers sets, using non-template specific control DNA; only in case of the DH2 / JH primer set a (sometimes faint) 340-350 bp product was observed in HeLa DNA. 20 Further sequencing revealed that this nonspecific product was due to false priming of the DH2 primer to a DNA sequence upstream of the JH4 segment. However, as the size of this nonspecific product was so different from the sizes of any of the true DH-JH PCR products, it was decided not to design a new DH2 primer. In fact, the nonspecific 350 bp band can be employed as an internal marker for successful DNA amplification and hence 25 the quality of the template DNA, being hardly or only faintly visible when enough clonal or polyclonal DH-JH template is available (e.g. in tonsillar DNA or DNA from particular leukemic samples), but being especially strong in samples containing low numbers of lymphoid cells with DH-JH rearrangements.

30 Serial dilutions of DNA from the clonal reference samples into tonsillar DNA generally resulted in sensitivities of 5% or lower (0.5-1% in case of the DH6-JH rearrangement) using HD analysis; sensitivities in GS analysis were generally 1-2 dilution steps better, i.e. 1% or lower. The clonal DH7-JH target could only be detected with a sensitivity of ~10%, which is most probably caused by primer consumption in PCR amplicons involving the non-rearranged germline DH7 and JH gene segments.

Although the initial multiplex strategy, as suggested from the OLIGO 6.2-assisted primer design, was to divide the various DH primers over two tubes, it was decided after testing various multiplex approaches to combine all primers into one multiplex tube (tube D of *IGH* clonality assay), except for the DH7 primer, which was 5 included in a separate tube (tube E of *IGH* clonality assay). The reason to exclude the DH7 primer was the complicated germline pattern, due to easy amplification of alleles with non-rearranged DH7 segments. Using this two-tube multiplex approach, all clonal reference samples were still detectable. Under multiplex conditions the detection limits for these various clonal targets were logically less optimal as compared to the single 10 assays, ranging from ~5% (DH3, DH4, and DH6) to ~10% (DH2, and DH5). For the DH1 clonal reference sample that was available, a sensitivity of ~20% was observed; at a later stage the DH1-JH rearrangement of cell line KCA was found to be detectable down to 15 10% in the multiplex assay. As tube E only contains the DH7 primer, the 10% sensitivity for this tube was the same as mentioned before. The same multiplex analysis performed with 500 ng instead of 100 ng DNA of the serial dilutions, resulted in slightly better sensitivities. The use of serial dilutions in MNC DNA instead of tonsillar DNA did not clearly affect detection limits of the assays for DH-JH recombinations.

Results of general testing phase

20 Following initial testing in the three laboratories involved in primer design, the developed *IGH* DH-JH multiplex PCR assay was further evaluated using the 90 Southern blot-defined samples. Every sample was analyzed in parallel in four laboratories by HD analysis and in five laboratories by GS analysis; in another two laboratories all samples 25 were analyzed by both techniques. All together a total of six HD and seven GS analysis results were obtained per sample per tube. Despite concordant results (> 80% of laboratories with identical results) in the vast majority of samples, nine showed inter-laboratory discordancies in tube D. Further analysis revealed that these could be explained by either the presence of a small clone with weak clonal products, or to large 30 size products (~390 and larger). In a few cases the products were so large, that only after sequencing it became clear that they concerned true but extended DH-JH rearrangements, either from upstream DH (e.g. DH6-25—DH1-26-JH in NL-12) or from downstream JH gene segments (e.g. DH6-25-JH4—JH5 in PT-14). In all three cases (NL-17, mycosis fungoides; FR-1, B-CLL; FR-5, FCL) in which clonal products were found using tube E, the results were completely concordant between laboratories.

When evaluating results from HD and GS analysis, it appeared that these were comparable, although in general the number of laboratories showing identical results was slightly higher upon HD as compared to GS analysis (Figure 4B and C).

Direct comparison of DH-JH multiplex PCR results with SB data is virtually impossible, as hybridization with a single probe (IGHJ6) in the JH region does not allow discrimination between VH-JH and DH-JH rearrangements. In three samples it was clear that detection of clonal products of the combined VH-JH and DH-JH assays did not fit with configuration of the *IGH* locus in SB analysis. Remarkably, no clonal DH-JH PCR products were observed in the pre-follicular B-cell malignancies. In contrast, 11/16 B-CLL samples and 12/25 (post-)follicular B-cell malignancy samples did contain clonally rearranged DH-JH PCR products. In three of the eighteen T-cell malignancy cases clonal DH-JH rearrangements were seen; these concerned T-LBL (ES-9) and mycosis fungoides (NL-17) cases with SB-detected *IGH* rearrangements, and a case of T-NHL/EATL (PT-4) without SB-detected *IGH* rearrangements, probably because of the low tumor load of <15%. All 15 reactive cases only showed polyclonal DH-JH PCR products, in accordance with SB results. In category D with difficult diagnoses, three samples (PT-12, GBS-10, and GBN-8) showed clonal *IGH* DH-JH PCR products, which was in line with SB data as well as *IGK* PCR data in two of three cases; in another two samples (PT-6 and GBS-9), both T-cell rich B-NHL cases, clonal DH-JH products were found in addition to clonal *IGK* and/or *IGL* products, but without evidence for clonality from SB analysis, which might best be explained by the small size of the B-cell clone in these samples.

In order to determine the additional value of DH-JH PCR analysis, the results were compared to those of VH-JH PCR analysis. In five (NL-4, PT-14, GBN-2, FR-7, NL-12) B-cell malignancies clonal DH-JH PCR products were found, whereas only polyclonal VH-JH PCR products were observed.

Conclusion

In conclusion, based on the initial and general testing phases, DH-JH PCR analysis appears to be of added value for clonality assessment. Although HD analysis results might be interpreted slightly more easily, there is no clear preference for either HD or GS analysis as they are both suitable for analyzing amplified PCR products. A potential difficulty in DH-JH PCR analysis is the relatively large size range of expected PCR products, due to scattered primer positions and to extended amplifications from upstream DH or downstream JH gene segments, implying that long runs are recommended for GS analysis. Finally, the remarkable position of the DH7-27 gene

segment in the *IGH* locus causes a ladder of germline amplification products in tube E, with clonal products being easily recognizable as much smaller bands / peaks.

EXAMPLE 3. *IGK* gene rearrangements: V_{κ} - J_{κ} , V_{κ} -Kde / intronRSS-Kde
Background

The human *IGK* light chain locus (on chromosome 2p11.2) contains many distinct V_{κ} gene segments, grouped into seven V_{κ} gene families, as well as five J_{κ} gene segments upstream of the C_{κ} region. Originally, the V_{κ} gene segments were designated according to the nomenclature as described by Zachau et al.⁶⁹ An alternative nomenclature groups the V_{κ} gene segments in seven families and is used in the ImMunoGeneTics database.⁴⁶ Here we follow the latter nomenclature. The $V_{\kappa}1$, $V_{\kappa}2$, and $V_{\kappa}3$ families are multi-member families including both functional and pseudo gene segments, whereas the other families only contain a single ($V_{\kappa}4$, $V_{\kappa}5$, $V_{\kappa}7$) or a few segments ($V_{\kappa}6$).⁷⁰ Remarkably, all V_{κ} gene segments are dispersed over two large duplicated clusters, one immediately upstream and in the same orientation as the J_{κ} segments, and the other more distal and in an inverted orientation (Figure 5A).⁷¹ The latter implies that so-called inversion rearrangements are required to form V_{κ} - J_{κ} joints involving V_{κ} genes of the distal cluster. In addition to the V_{κ} and J_{κ} segments, there are other elements in the *IGK* locus that can be involved in recombination. The kappa deleting element (Kde), approximately 24 kb downstream of the J_{κ} - C_{κ} region, can rearrange to V_{κ} gene segments (V_{κ} -Kde), but also to an isolated RSS in the J_{κ} - C_{κ} intron (intronRSS-Kde).^{24,72} Both types of rearrangements lead to functional inactivation of the *IGK* allele, through deletion of either the C_{κ} exon (intronRSS-Kde rearrangement) or the entire J_{κ} - C_{κ} area (V_{κ} -Kde rearrangement).

As human *IGK* recombination starts in precursor B-cells in the bone marrow, *IGK* rearrangements can also be detected in precursor B-cell acute leukemias (30-45% of alleles, depending on age). Although V_{κ} - J_{κ} joinings are present, these *IGK* rearrangements mainly concern recombinations involving Kde (25-35% of alleles). In childhood precursor B-ALL V_{κ} -Kde recombination predominates over intron-Kde, whereas in adult ALL the deletions exclusively concern V_{κ} -Kde couplings.^{24,73,74} In chronic B-cell leukemias *IGK* rearrangements are even more frequent, being detectable on 75% ($Ig\kappa^+$ cases) or even 95% ($Ig\lambda^+$ cases) of all *IGK* alleles. By definition, functional V_{κ} - J_{κ} rearrangements are found on at least one allele in $Ig\kappa^+$ B-cell leukemias; the non-coding second allele is either in germline configuration, or inactivated through V_{κ} -Kde (8% of alleles) or intronRSS-Kde (8% of alleles) recombination. Kde rearrangements are frequent in $Ig\lambda^+$ B-cell leukemias (~85% of alleles), with a slight predominance of intronRSS-Kde recombinations over V_{κ} -Kde rearrangements. This implies that virtually all $Ig\lambda^+$ leukemias contain a Kde rearrangement, while potentially functional V_{κ} - J_{κ}

is almost identical between various normal and malignant B-cell populations and largely reflects the number of available gene segments within each family. Both in $V\kappa$ - $J\kappa$ as well as in $V\kappa$ -Kde rearrangements, $V\kappa$ gene segments from the first four families ($V\kappa 1$ to $V\kappa 4$) predominate. $V\kappa 2$ gene usage appeared to be higher in precursor B-ALL than in more mature B-cell lymphoproliferations or normal B cells. Remarkably, the distal inverted $V\kappa$ cluster was rarely used in $V\kappa$ - $J\kappa$ rearrangements, whereas $V\kappa$ pseudogene segments were never involved, also not in $Ig\lambda^+$ cases.⁷⁶ Little is known about $J\kappa$ gene segment usage, but sparse data show that $J\kappa 1$, $J\kappa 2$, and $J\kappa 4$ are the most frequently used $J\kappa$ gene segments.⁷⁵

$V\kappa$ - $J\kappa$ rearrangements can be important complementary PCR target for those types of B-cell proliferations in which somatic hypermutations may hamper amplification of the VH - JH target, but recombinations involving Kde are probably even more valuable. Deletion of intervening sequences in the $J\kappa$ - $C\kappa$ intron results in the removal of the IGK enhancer, which is thought to be essential for the somatic hypermutation process to occur. Rearrangements involving Kde are therefore assumed to be free of somatic hypermutations, and hence should be amplified rather easily.

Primer design

Using OLIGO 6.2 software, six family-specific $V\kappa$ primers were designed to recognize the various $V\kappa$ gene segments of the seven $V\kappa$ families; the $V\kappa 6$ family gene segments were covered by the $V\kappa 1$ family primer (Figure 5B). In case of the relatively large $V\kappa 1$, $V\kappa 2$, and $V\kappa 3$ families only the functional $V\kappa$ gene segments were taken into consideration, as the less homologous pseudo gene segments complicated optimal primer design too much. The family-specific $V\kappa$ primers were designed to be used in combination with either a set of two $J\kappa$ primers ($J\kappa 1$ -4, covering the first four $J\kappa$ segments and $J\kappa 5$ covering the fifth) or a Kde primer (Figure 5B). For analysis of Kde rearrangements an additional forward primer recognizing a sequence upstream of the intronRSS was made. In order to show minimal cross-annealing to other $V\kappa$ family segments and still be useful in multiplex reactions, the various primers could not be designed at similar positions relative to RSS elements (Figure 5B). The expected PCR product sizes of $V\kappa$ - $J\kappa$ joints range from ~115-135 bp (for $V\kappa 7$ - $J\kappa$ joints) to ~280-300 bp ($V\kappa 2$ - $J\kappa$ rearrangements). For the Kde rearrangements, product size ranges are from ~195-215 bp ($V\kappa 7$ -Kde) to ~360-380 bp ($V\kappa 2$ -Kde), whereas the intronRSS-Kde products are ~275-295 bp.

Results of initial testing phase

For initial testing of the individual primers, several cell lines and patient samples with precisely defined clonal $V\kappa$ - $J\kappa$, or $V\kappa$ -Kde / intronRSS-Kde rearrangements were used. The patient samples with $V\kappa$ - $J\kappa$ joints mostly concerned chronic B-cell leukemias, which were additionally selected on basis of a high tumor load for easy and sensitive detection of the involved rearrangement. Unfortunately, clonal reference samples were not available for all $V\kappa$ - $J\kappa$ targets; especially the more rare types of rearrangements involving $V\kappa$ 5, $V\kappa$ 7 and/or $J\kappa$ 5 were not represented in the series of reference samples. For these targets and also for the targets for which clonal reference samples were available, healthy control tonsillar or MNC DNA samples were employed, in which PCR products of the correct expected sizes were indeed observed. The only exception was the $V\kappa$ 7 / $J\kappa$ 5 primer combination; most probably $V\kappa$ 7- $J\kappa$ 5 joinings are so rare in normal B cells, that these PCR products were hardly or not detectable in tonsils. Rearranged products within the expected size ranges could be detected in all clonal reference samples, under standard PCR conditions using 1.5 mM MgCl₂ and either ABI Gold Buffer or ABI Buffer II. However, in a few cases weak amplification of particular $V\kappa$ - $J\kappa$ rearrangements was observed with other $V\kappa$ family / $J\kappa$ primer sets, due to slight cross-annealing of the $V\kappa$ 3 primer to a few $V\kappa$ 1 gene segments. Furthermore, in a few of the clonal reference samples clear additional clonal PCR products were seen with other $V\kappa$ / $J\kappa$ or even $V\kappa$ / Kde and intronRSS / Kde primer sets; in most samples this could be explained by the complete configuration of the two *IGK* alleles. This occurrence of multiple clonal PCR products illustrates the complexity of *IGK* rearrangement patterns in a given cell sample, mainly caused by the potential occurrence of two clonal rearrangements on one allele ($V\kappa$ - $J\kappa$ and intron RSS-Kde). This complexity does not hamper but support the discrimination between polyclonality and monoclonality.

No nonspecific annealing of the primers was observed for any of the $V\kappa$ - $J\kappa$ and $V\kappa$ -Kde / intron RSS-Kde primer sets, when using HeLa DNA as a non-template specific control. Serial dilutions of DNA from the clonal reference samples into tonsillar DNA generally resulted in sensitivities of 5-10 % for $V\kappa$ - $J\kappa$ rearrangements and 1-10 % for $V\kappa$ -Kde rearrangements, using HD analysis. In general, the sensitivities in GS analysis were approximately one dilution step better. The only slightly problematic target was the intronRSS-Kde target that could only be detected down to the 10% serial dilution in the employed patient sample. This is probably caused by the fact that intronRSS-Kde rearrangements are abundant in DNA from both Ig κ ⁺ and Ig λ ⁺ tonsillar B cells, which were used in the dilution experiments.

of two different multiplex PCR reaction tubes. In the $V\kappa$ - $J\kappa$ tube (tube A) all $V\kappa$ primers were combined with both $J\kappa$ primers, whereas tube B contained all $V\kappa$ primers plus the intronRSS primer in combination with the Kde reverse primer (Figure 5B). All 5 beforementioned clonal reference samples were detectable using this two-tube multiplex approach. Of note is the observation that in non-clonal tonsil samples a predominant, seemingly clonal band of ~150 bp was detected using the $V\kappa$ - $J\kappa$ multiplex tube A analysis. The presence of this product, which is seen in HD analysis but especially in GS analysis, can be explained by the limited heterogeneity of $V\kappa$ - $J\kappa$ junctional regions 10 leading to a high frequency of products of an average size of ~150 bp. Furthermore, in some samples a sometimes weak 404 bp nonspecific band was observed in tube B. Although sensitivities were on average slightly better in other multiplex approaches in which the $V\kappa$ primers were further subdivided over multiple tubes, the feasibility of having only two tubes to analyze all relevant *IGK* rearrangements, finally was the most 15 important argument for choosing the two-tube multiplex strategy as given in Figure 5B. Detection limits for the various clonal targets in the two-tube multiplex approach were ~10% for most of the clonal $V\kappa$ - $J\kappa$ rearrangements ($V\kappa 1$ - $J\kappa 4$, $V\kappa 2$ - $J\kappa 4$, $V\kappa 3$ - $J\kappa 4$) derived from informative samples with a high tumor load; several of the $V\kappa$ -Kde targets were 20 detectable with a still reasonable sensitivity of ~10%, but a few other samples containing $V\kappa 2$ -Kde, $V\kappa 5$ -Kde, and also intronRSS-Kde targets showed detection limits above 10%. Even the use of 500 ng serially diluted DNA instead of 100 ng hardly resulted in better 25 sensitivities, whereas serial dilutions in MNC DNA did not affect the detection limits either. Nevertheless, detection limits of serial dilutions of reference DNA in water were all in the order of 0.5-1 %, which shows that the chosen multiplex *IGK* PCR assay as such is good. It is important to note that potential clonal cell populations in lymph nodes or peripheral blood in practice will have to be detected within a background of polyclonal 30 cells, which can hamper sensitive clonality detection, especially in samples with a relatively high background of polyclonal B-cells.

30 Results of general testing phase

Following initial testing in the four laboratories involved in primer design, the developed *IGK* multiplex PCR assay was further evaluated using 90 Southern blot-defined samples. Every sample was analyzed in parallel via HD (five laboratories) and GS (two laboratories) analysis; in another four laboratories all samples were analyzed by both 35 techniques. Taken together, eight HD and five GS analysis results were available per

sample per tube. In the vast majority of samples >80% of laboratories produced identical results, i.e. either clonal bands / peaks or polyclonal smears / curves in one or both tubes. However, in nine (~10%) samples discordancies were found between laboratories, which remained after repetitive analysis of these samples. More detailed analysis revealed that 5 in at least six cases the approximately 150 and 200 bp sizes of the clonal products in tube A could not easily be discriminated from polyclonal products of roughly the same size. This is an inherent difficulty in especially $V\kappa$ - $J\kappa$ analysis, which is caused by the relatively limited junctional heterogeneity of these rearrangements. In two samples the results from tube B were however so clear in all laboratories with both techniques that 10 in fact no discrepancy prevailed. In one sample (ES-8) a large product of around 500 bp appeared to be the reason for discrepant inter-laboratory results; further sequencing revealed that amplification starting from the downstream $J\kappa$ segment caused production of an extended $V\kappa$ 1- $J\kappa$ 3- $J\kappa$ 4 PCR product.

When evaluating results from HD and GS analysis, it appeared that these were 15 rather comparable, although in general the number of laboratories showing identical results was slightly higher upon HD as compared to GS analysis (Figure 5C and D). Remarkably, in one sample (GBS-4) HD analysis revealed a clear product in both tubes, whereas GS analysis only showed polyclonality. Cloning of the HD product showed a 20 peculiar $V\kappa$ 3- $V\kappa$ 5 PCR product, which was not observed in any other sample; the $V\kappa$ - $V\kappa$ configuration of this product explained why it was not detected with labeled $J\kappa$ primers in GS analysis.

Comparison of PCR results with SB data revealed no SB-PCR discrepancies in the pre-follicular B-cell malignancies and B-CLL samples; in line with the presence of rearranged *IGK* bands in SB analysis, all samples contained clonal *IGK* PCR products. 25 In contrast, in the 25 (post-)follicular B-cell malignancy samples clonal *IGK* PCR products were missed in four DLCL cases (ES-5, PT-13, PT-14, FR-7) and one PC leukemia (NL-19) with both techniques and in another DLCL case (GBS-4, see above) with GS analysis only. In all cases this was most probably caused by somatic hypermutation. Interestingly, in one FCL case (NL-4), a clonal PCR product was found, 30 whereas SB analysis revealed a germline band in case of the *IGK* genes and weak clonal bands upon *IGH* analysis. In all 18 T-cell malignancy cases and all 15 reactive cases (category C) polyclonal *IGK* PCR products were found in accordance with SB results, except for one peripheral T-NHL case (FR-10). Next to the clonal TCR and *IGK* products this sample also showed clonal *IGH* and *IGL* PCR products, but no clonal Ig 35 rearrangements in SB analysis, probably reflecting the presence of a small additional B-

samples (GBS-10 and GBN-8) showed clonal *IGK* PCR products, in line with SB data; however, in another two samples (PT-6 and GBS-9), both T-cell rich B-NHL cases, clonal *IGK* PCR products were found as well as clonal *IGH* and/or *IGL* products, but without evidence for clonality from SB analysis. Also this discrepancy can probably be explained by the small size of the B-cell clone in these two patient samples.

To determine the additional value of analyzing the *IGK* locus, we compared the results of *IGK* PCR analysis to those of *IGH* PCR analysis. In five (ES-2, NL-4, PT-8, GBN-2, ES-8) of the nine samples in which no clonal VH-JH PCR products were found, 10 clonal products were readily observed in *IGK* analysis. When taking into account both VH-JH and DH-JH analysis, *IGK* PCR analysis was still complementary to *IGH* PCR analysis in three of these cases in detecting clonal Ig PCR products.

Conclusion

15 In conclusion, based on the initial and general testing phases as well as preliminary evidence from use of these multiplex assays in pathologically well-defined series of lymphoproliferations, PCR analysis of the *IGK* locus has clear (additional) value for clonality detection. Nevertheless, care should be taken with interpretation of seemingly clonal bands in especially tube A, due to the inherent restricted *IGK* 20 junctional heterogeneity. As this problem is especially apparent in GS analysis, HD analysis is slightly preferred over GS analysis, although it should be marked that in some cases GS analysis may facilitate proper interpretation of results. Another potential pitfall is the relatively large size range of expected rearranged *IGK* products, due to 25 scattered primer positions, and to extended amplifications from downstream J κ gene segments. This implies that long runs are recommended for GS analysis. Finally, the inherent complexity of multiple rearrangements in the *IGK* locus (V κ -J κ and Kde rearrangements on the same allele), together with a low level of cross-annealing of V κ primers, may occasionally result in patterns with multiple bands or peaks, resembling oligoclonality. However, with these considerations in mind, the two-tube *IGK* multiplex 30 PCR system can be valuable in PCR-based clonality diagnostics.

EXAMPLE 4. *IGL* gene rearrangements

Background

IGL gene rearrangements are present in 5 to 10% of Ig κ ⁺ B-cell malignancies and in all Ig λ ⁺ B-cell malignancies.⁷⁵ Therefore V λ -J λ rearrangements potentially represent an attractive extra PCR target for clonality studies to compensate for false-negative *IGH* VH-JH PCR results, mainly caused by somatic mutations. The *IGL* locus spans 1Mb on chromosome 22q11.2.⁷⁷⁻⁷⁹ There are 73-74 V λ genes over 900 kb, among which 30-33 are functional (Figure 6A). Upon sequence homology, the V λ genes can be grouped in 11 families and three clans. Members of the same family tend to be clustered on the chromosome. The J λ and C λ genes are organized in tandem with a J λ segment preceding a C λ gene. Typically there are 7 J-C λ gene segments, of which J-C λ 1, J-C λ 2, J-C λ 3, and J-C λ 7 are functional and encode the four Ig λ isotypes (Figure 6A).^{80, 81} There is however a polymorphic variation in the number of J-C λ gene segments, since some individual may carry up to 11 of them, due to an amplification of the C λ 2-C λ 3 region.^{82, 83}

Several studies have shown that the *IGL* gene repertoire of both normal and malignant B cells is biased.^{48, 49, 84, 85} Thus over 90% of V λ genes used by normal B cells belong to the V λ 1, V λ 2 and V λ 3 families, which comprise 60% of the functional genes. Moreover, three genes (2-14, 1-40, 2-8) account for about half of the expressed repertoire. While normal B cells use J-C λ 1, J-C λ 2 and J-C λ 3 gene segments in roughly equivalent proportions, neoplastic B cells tend to use predominantly J-C λ 2 and J-C λ 3 gene segments.⁴⁹ In both normal and malignant B cells the J-C λ 7 is used very rarely (1%). This latter finding was however challenged by a single-cell study of normal cells which found that more than half of the rearrangements employed the J-C λ 7 gene segments.⁸⁶ In contrast to the mouse, there is some junctional diversity due to exonuclease activity and N nucleotide addition in human *IGL* gene rearrangements.^{82, 85-87} It is however much less extensive than that of the *IGH* locus, and a number of rearrangements result from the directly coupling of germline V λ and J λ gene segments. Nevertheless, the *IGL* locus might represent an alternative complementary locus to *IGH* for B-cell clonality studies.

Primer design

Considering the biased V λ repertoire, we chose to amplify only rearrangements which used the V λ 1, V λ 2 and V λ 3 gene segments. A single consensus primer recognizing both V λ 1 and V λ 2 gene segments, as well as a V λ 3 primer, were designed in regions of high homology between members of the same family (Figure 6B). Initial experiments showed that they worked as well in multiplex as separately. In fact, cross annealing of

V λ 3 primer hybridizing to some V λ 1 or V λ 2 genes (or vice versa) could be observed when V λ primers were used separately; it was not seen however in multiplex PCR.

A single consensus primer was designed for the J λ 1, J λ 2 and J λ 3 gene segments and has one mismatch in its central portion compared to each of the germline sequences.

5 In preliminary experiments it was found to give rather better results than a combination of perfectly matched J λ 1 and J λ 2-J λ 3 primers. Since a single study reported the frequent usage of the J λ 7 gene in normal B cells,⁸⁸ we also designed a J λ 7 specific primer. When tested on various polyclonal B cell samples, we could hardly detect any signal in HD analysis, in contrast to amplifications performed on the same samples using the J λ 1,
10 J λ 2-J λ 3 or the J λ consensus primers. Similarly, we could not detect any rearrangement with this primer when analyzing a collection of monoclonal B-cell tumors. Based on these results as well as the other reports in the literature ⁸⁹, we concluded that the non-confirmed high frequency of J λ 7 rearrangements (in a single study) ⁸⁸had been caused by a technical pitfall and consequently, we decided not to include the J λ 7 primer. The PCR
15 assay for the detection of *IGL* gene rearrangements in clonality study therefore consists of a single tube containing three primers (Figure 6B). This single tube was expected to detect the vast majority of the rearrangements.

Results of initial testing phase

20 Initial testing on a set of monoclonal and polyclonal samples showed they could very well be differentiated upon HD analysis of PCR products on 10% polyacrylamide gel electrophoresis (Figure 6C). Clonal *IGL* rearrangements were seen in the homoduplex region, with one or sometimes two weaker bands in the heteroduplex region, while polyclonal rearrangements appeared as a smear in the heteroduplex region (Figure 6C).

25 Nonspecific bands were not observed. It should be noted that because of the limited size of the junctional region, it is extremely difficult to distinguish polyclonal from monoclonal rearrangements by running a simple polyacrylamide gel without performing a heteroduplex formation. Along this line, analysis of PCR products by GS proved to be less straightforward (Figure 6C). While monoclonal rearrangements were clearly
30 identified, the polyclonal rearrangement pattern had an oligoclonal aspect due to the limited junctional diversity. The interpretation was more difficult, particularly to distinguish polyclonal cases from those with a minor clonal B-cell population in a background of polyclonal B-cells. We therefore recommend HD analysis as the method of choice to analyze *IGL* gene rearrangements.

The sensitivity of the assay, performed on several cases, proved to be about 5% (2.5% - 10%) when dilution of tumor DNA was done in PB-MNC and about 10% (5% - 20%) when diluted in lymph node DNA.

5 Results of general testing phase

The single-tube *IGL* PCR assay was evaluated on the series of 90 Southern blot defined lymphoid proliferations. This testing was done by nine laboratories, four with HD analysis only, one with GS analysis only, and four using both techniques. Clonal *IGL* gene rearrangements were detected in 19 cases. In 15 of them more than 70% 10 concordance was obtained within the nine laboratories. In four cases less than 70% concordancy was obtained, which could be explained by minor clonal *IGL* gene rearrangements in three of them (ES-12, GB-10, and FR-10). This discordancy in the fourth case (PT-11) remains unexplained, particularly because no *IGL* gene rearrangements were detected by Southern blotting. As concluded from the initial 15 testing, interpretation of GS analysis was more difficult than HD analysis, especially in the case of minor clonal populations. Of these 19 clonal *IGL* gene cases, 17 were B-cell proliferations (16 mature and one precursor B-cell). One case (ES12) corresponded to Hodgkin's disease and another (FR-10) to a T-NHL. Both had only a minor clonal *IGL* gene rearrangement, and FR-10 also displayed a clonal *IGK* gene rearrangement.

20 Comparison with Southern blot data showed some discrepancies. Six cases with clonal *IGL* gene rearrangements by PCR appeared as polyclonal by Southern blot analysis. Three of them (PT-6, ES-12, FR-10) concerned minor clonal populations which may have been below the sensitivity level of the Southern blot technique. In the three other cases (NL-19, ES-1, PT-11) a clonally rearranged band may have been missed by 25 the fairly complex rearrangement pattern of the *IGL* locus on Southern blot.^{28, 49} Conversely the PCR assay failed to detect clonal rearrangements which were seen by Southern blot analysis in two cases (GBS-6, FR-5). However these were follicular lymphomas in which a high degree of somatic hypermutations may have prevented annealing of the *IGL* gene primers.

30

Conclusion

In conclusion, a single-tube PCR assay for the detection of *IGL* gene rearrangements containing only three primers (Figure 6B) allows to detect the vast majority of *IGL* gene rearrangements (V λ 1, V λ 2, and V3 gene rearrangements). Heteroduplex analysis is the 35 preferred analytic method, though GeneScan analysis can be used, but maximal caution

is recommended to avoid overinterpretation of clonality due to the limited junctional diversity.

5 **EXAMPLE 5: *TCRB* gene rearrangements: V β -J β , D β -J β**

Background

Molecular analysis of the *TCRB* genes is an important tool for assessment of clonality in suspect T-cell proliferations. *TCRB* gene rearrangements occur not only in almost all mature T-cell malignancies but also in about 80% of the CD3 negative T-cell acute lymphoblastic leukemias (T-ALL) and 95% of the CD3 positive T-ALL.²⁸ *TCRB* rearrangements are not restricted to T-lineage malignancies as about one third of precursor-B-ALL harbor rearranged *TCRB* genes.²⁹ Their frequency is much lower (0 to 7%) in mature B cell proliferations.²¹

The human *TCRB* locus is located on the long arm of chromosome 7, at band 7q34 and spans a region of 685 kb. In contrast to the *TCRG* and *TCRD* loci the V region gene cluster of the *TCRB* locus is far more complex (Figure 7A).¹ It contains about 65 V β gene elements for which two different nomenclatures are used: the one summarized by Arden et al.⁵⁰ follows the gene designation of Wei et al.⁵⁸ and groups the V β genes into 34 families. The alternative nomenclature proposed by Rowen et al.⁵¹ subdivides 30 V β gene subgroups and was later adopted by IMGT, the international ImMunoGeneTics database <http://imgt.cines.fr> (initiator and coordinator: Marie-Paule Lefranc, Montpellier, France). [Lefranc, 2003 #212; Lefranc, 2003 #219] The largest families, V β 5, V β 6, V β 8 and V β 13 (Arden nomenclature) reach a size of seven, nine, five and eight members, respectively. Twelve V β families contain only a single member. In general, the families are clearly demarcated from each other.⁵⁰ In this report we follow the Arden nomenclature.⁵⁰

39-47 of the V β gene elements are qualified as functional and belong to 23 families. 7-9 of the nonfunctional V β elements have an open reading frame but contain alterations in the splice sites, recombination signals and/or regulatory elements. 10-16 are classified as pseudogenes. In addition, a cluster of six non-functional orphan V β genes have been reported that are localized at the short arm of chromosome 9 (9p21).^{59, 60} They are not detected in transcripts.^{50, 51}

All but one V β genes are located upstream of two D β -J β -C β clusters. Figure 7A illustrates that both C β gene segments (C β 1 and C β 2) are preceded by a D β gene (D β 1 and D β 2) and a J β cluster which comprises six (J β 1.1 to J β 1.6) and seven (J β 2.1 to J β 2.7)

functional $\text{J}\beta$ segments. $\text{J}\beta$ region loci are classified into two families according to their genomic localization, not to sequence similarity.^{51, 88, 91}

Due to the large germline encoded repertoire, the combinatorial diversity of *TCRB* gene rearrangements is extensive compared to the *TCRG* and *TCRD*

5 rearrangements. The primary repertoire of the $\text{TCR}\beta$ molecules is further extended by an addition of an average of 3.6 (V-D junction) and 4.3-(D-J junction) nucleotides and deletion of an average of 3.6 (V), 3.8 (5' of D), 3.7 (3' of D) and 4.1 (J) nucleotides.⁵¹ The complete hypervariable region resulting from the junction of the V, D and J segments comprises characteristically nine or ten codons. Size variation is limited, as 7 to 12
10 residues account for more than 80% of all functional rearrangements in contrast to the broad length repertoire of the *IGH* CDR3 region.⁹²

During early T-cell development the rearrangement of the *TCRB* gene consists of two consecutive steps: $\text{D}\beta$ to $\text{J}\beta$ rearrangement and $\text{V}\beta$ to $\text{D-J}\beta$ rearrangement with an interval of one to two days between these two processes.⁹³ The $\text{D}\beta 1$ gene segment may
15 join either $\text{J}\beta 1$ or $\text{J}\beta 2$ gene segments but the $\text{D}\beta 2$ gene segment generally joins only $\text{J}\beta 2$ gene segments because of its position in the *TCRB* gene locus.^{28, 51} Due to the presence of two consecutive *TCRB* D-J clusters, it is also possible that two rearrangements are
detectable on one allele: an incomplete *TCRB* $\text{D}\beta 2\text{-J}\beta 2$ rearrangement in addition to a
complete or incomplete rearrangement in the *TCRB* $\text{D}\beta 1\text{-J}\beta 1$ region.¹

20 In *TCRB* gene rearrangements, a non-random distribution of gene segment usage is seen. In healthy individuals, some $\text{V}\beta$ families predominate in the peripheral blood T-cell repertoire (e.g. $\text{V}\beta 1\text{-V}\beta 5$), while others are only rarely used (e.g. $\text{V}\beta 11$, $\text{V}\beta 16$, $\text{V}\beta 18$, $\text{V}\beta 23$). Mean values of the $\text{V}\beta$ repertoire seem to be stable during aging, although the standard deviation increase in the elderly.^{13, 94} Also in the human thymus some $\text{V}\beta$ gene
25 segments dominate: the most prevalent seven $\text{V}\beta$ genes ($\text{V}\beta 3\text{-}1$, $\text{V}\beta 4\text{-}1$, $\text{V}\beta 5\text{-}1$, $\text{V}\beta 6\text{-}7$, $\text{V}\beta 7\text{-}2$, $\text{V}\beta 8\text{-}2$, $\text{V}\beta 13\text{-}2$) cover nearly half of the entire functional *TCRB* repertoire.⁹⁵ The representation of J segments is also far from even. The $\text{J}\beta 2$ family is used more frequently than the $\text{J}\beta 1$ family (72% vs. 28% of *TCRB* rearrangements).⁹⁶ In particular, the proportion of $\text{J}\beta 2.1$ is higher than expected (24%) followed by $\text{J}\beta 2.2$ (11%) and $\text{J}\beta 2.3$
30 and $\text{J}\beta 2.5$ (10% each).⁹⁵

35 *TCRB* gene rearrangement patterns differ between categories of T cell malignancies. Complete *TCRB* $\text{V}\beta\text{-J}\beta 1$ rearrangements and incompletely rearranged alleles in the *TCRB* $\text{D}\beta\text{-J}\beta 2$ cluster are seen more frequently in $\text{TCR}\alpha\beta^+$ T-ALL as compared to $\text{CD}3^+$ T-ALL and $\text{TCR}\gamma\delta^+$ T-ALL.²⁸ In the total group of T-ALL the *TCRB* $\text{D}\beta\text{-J}\beta 1$ region is relatively frequently involved in rearrangements in contrast to cross-

The development of monoclonal antibodies against most V β domains has helped to identify V β family expansions.¹³ However, TCR gene rearrangement analysis is essential for clonality assessment in T cell lymphoproliferative disorders. As the restricted germline encoded repertoire of the *TCRG* and *TCRD* loci facilitates DNA based PCR approaches, various PCR methods have been established for the detection of *TCRG* and *TCRD* gene rearrangements.⁹⁷⁻⁹⁹ Nevertheless, the limited junctional diversity of *TCRG* rearrangements leads to a high background amplification of similar rearrangements in normal T cells (Example 6). The *TCRD* gene on the other hand is deleted in most mature T cell malignancies.²¹ Therefore DNA based *TCRB* PCR techniques are needed for clonality assessment. In addition, *TCRB* rearrangements are of great interest for follow-up studies of lymphoproliferative disorders, because the extensive combinatorial repertoire of *TCRB* rearrangements and the large hypervariable region enables a highly specific detection of clinically occult residual tumor cells. However, the extensive germline encoded repertoire renders PCR assays more difficult. Some published PCR approaches use the time consuming procedure of multiple tube approaches with a panel of family- or subfamily-specific primers.^{96, 100} Usage of highly degenerated consensus primers limits the number of detectable rearrangements that are theoretically covered by the primers because there is no single common sequence of sufficient identity to allow a reliable amplification of all possible rearrangements.^{42, 101, 102} Some published assays use a nested PCR requiring an additional PCR reaction.^{42, 102} Other assays focus on analysis of the *TCRB* V β -D β -J β -C β transcripts to limit the number of primers needed.^{16, 100, 103} However, a major drawback of these mRNA based approaches is the need for fresh or frozen material and a reverse transcription step before the PCR amplification.

We tried to overcome these limitations by creating a completely new and convenient DNA based *TCRB* PCR. We designed multiple V β and J β primers, covering all functional V β and J β gene segments and being suitable for combination in multiplex PCR reactions. In addition the assay is applicable for HD and GS analysis and also detects the incomplete *TCRB* D β -J β rearrangements with the same set of J β primers. In order to avoid problems due to cross priming we decided to design all V β and J β primers at the same conserved region of each gene segment.

Primer design

Initially a total of 23 V β , 2 D β (D β 1 and D β 2) and 13 J β (J β 1.1 to 1.6 and J β 2.1 to 2.7) primers were designed with all the V β and J β primers positioned in the same conserved region of each V β and J β gene segment so that the effects of cross-annealing in 5 a multiplex reaction could be neglected. In addition, rare polyclonal *TCRB* V-J rearrangements would not be mistaken for a clonal rearrangement even if they do not produce a fully polyclonal Gaussian peak pattern, because PCR products of all possible rearrangements are situated in the same size range.

For primer design, the rearrangeable pseudogenes or open reading frame genes 10 with alterations in splicing sites, recombination signals and/or regulatory elements or changes of conserved amino acids were taken into consideration whenever possible. However, the main objective was to cover all functional V β genes. The priming efficiency of each V β primer was checked for every V β gene using OLIGO 6.2 software. This led to 15 primers that were not strictly V β family specific and some of which covered V β gene segments of more than one family (Figure 7B). Since the 13 J β primers annealed to the same segment of each J β gene primer, dimerization made it necessary to split the J primers into two tubes. Initially, it was planned to use the primers in four sets of 20 multiplex reactions as follows: all 23 V β primers in combination with the six J β 1 family primers (240-285 bp), all 23 V β primers with the seven J β 2 family primers (240-285 bp), D β 1 (280-320 bp) with the six J β 1 primers, and D β 1 (280-320 bp) plus D β 2 (170-210 bp) 25 with the seven J β 2 family primers.

Results of initial testing phase

Initial monoplex testing of each possible primer combination was done using samples 25 with known monoclonal *TCRB* rearrangements and polyclonal controls. PCR products of the expected size range were generated with differences in product intensity and signal profile for polyclonal samples depending on the frequency of usage of distinct V β and J β gene segments. However, when the primers were combined in a multiplex reaction some J β 2 rearrangements in particular were missed and nonspecific products in the tubes B 30 and D were observed. In addition cross-priming between the J β 1 and J β 2 primers resulted in interpretation problems. As a consequence the J β 2 primers had to be redesigned and the primer combinations in the different tubes had to be rearranged: J β primers J β 2.2, 2.6 and 2.7 were slightly modified and added to tube A. The localization of 35 the primers J β 2.1, 2.3, 2.4 and 2.5 was shifted by 4 bp downstream to avoid primer dimerization and cross priming with the remaining J β primers. Only nonspecific bands

bp, 221 bp) and tube C (128 bp, 337 bp) using specific template DNA. However, because all nonspecific amplification products were outside the size ranges of the *TCRB* specific products, they did not affect interpretation and were considered not to be a problem.

5 However, using nonspecific template controls one additional faint 273 bp aspecific peak in tube A was visible by GS analysis. This product is completely suppressed when the DNA contains enough clonal or polyclonal *TCRB* rearrangements but can appear in samples comprising low numbers of lymphoid cells. In the initial testing phase relatively faint V-D-J PCR products were generated. Thus we optimized PCR conditions for
10 complete V-D-J rearrangements by increasing MgCl₂ concentration and the amount of Taq polymerase. Also usage of highly purified primers and application of ABI Buffer II instead of ABI Gold Buffer turned out to be very important. For detection of the incomplete D β -J β rearrangements, it was finally possible to mix all J β primers into one tube without loss of sensitivity or information. Consequently, the total number of
15 multiplex reactions could be reduced to three tubes.

The finally approved primer set is (Figure 7B):

tube A: 23 V β primers and 9 J β primers: J β 1.1-1.6, 2.2, 2.6 and 2.7

tube B: 23 V β primers and 4 J β primers: J β 2.1, 2.3, 2.4 and 2.5

20 **tube C:** D β 1, D β 2 and all 13 J β primers.

As tubes A and C contain J β 1 and J β 2 primers, differential labeling of J β 1 and J β 2 primers with different dyes (TET for J β 1.1-1.6 and FAM for J β 2.1-2.7 primers) allows GS discrimination of J β 1 or J β 2 usage in tube A and C reactions (see Figure 13A).

25 Sensitivity testing was performed via dilution experiments with various cell lines and patient samples with clonally rearranged *TCRB* genes in MNC. Single PCR dilution experiments generally reached sensitivity levels of at least 0.1% to 1%. As expected, the sensitivity decreased in multiplex testing, probably due to an increase of background amplification. Especially in GS analysis this background hampered interpretation due to
30 the relative small length variation of the *TCRB* PCR products. Nevertheless, in 40 of 46 positive controls tested a sensitivity of at least 1% to 10% was reached using heteroduplex or GeneScanning (Table 6).

Results of general testing phase

Eleven groups participated in the analysis of DNA from a series of 90 Southern blot-defined malignant and reactive lymphoproliferative disorders using the *TCRB* multiplex protocol. Every sample was analysed by HD in two laboratories and in six laboratories

5 using GS analysis. Another three laboratories used both techniques for PCR analysis (Figure 7C, D, and E). This testing phase as well as experience from use of these *TCRB* PCR assays raised some general issues about the protocol that were in part already described in the initial testing phase: 1. The limited length variation of the *TCRB* PCR products may hamper GS detection of clonal signals within a polyclonal background. 2. 10 Only bands/peaks within the expected size range represent clonal *TCRB* gene rearrangements. Especially for tube A a nonspecific control DNA must be included to define the aspecific 273 bp peak that may occur in situations without competition. 3. It is extremely important to use highly purified primers and ABI Buffer II (and not ABI Gold Buffer) for good PCR results as well as the recommended PCR product preparation 15 protocol for HD analysis. Of the 90 Southern blot-defined cases submitted, 29 were SB positive for monoclonal *TCRB* rearrangements. 25 of these clonal rearrangements (86%) were also detectable by the *TCRB* PCR. 23 rearrangements were disclosed by GS and HD analysis, two additional cases only by HD. One of the GS negative HD positive cases (FR-9) was interpreted as monoclonal on GS analysis by four of the nine laboratories 20 involved in the general testing phase (Figure 7C). However, due to a significant polyclonal background, interpretation of the GS patterns was difficult in this particular case. The other GS negative HD positive case displayed an atypical PCR product in tube C with a size of about 400 bp (Figure 7E). The PCR product was clearly visible in agarose gels and HD analysis but not by GS. After DNA sequencing of this fragment a *TCRB* 25 D β 1-D β 2 amplification product was identified explaining the unlabelled PCR product. Four SB positive cases (NL-15, NL-16, GBN-2 and FR-6) were neither detected by GS nor by HD analysis all of them with an underlying B lymphoid malignancy. Possible explanations for this failure are atypical rearrangements (e.g. incomplete V β -D β rearrangements),^{28, 104} sequence variations of the rearranged V β gene segments⁵¹ or a lack 30 of sensitivity for particular rearrangements.

62 of the samples were considered to be polyclonal by SB. For 61 (98%) of these cases PCR results were concordant with at least one method of analysis, for 57 (92%) cases results were concordant using both methods. The one SB negative sample (ES-14) found 35 to be monoclonal by HD and GS analysis showed an incomplete D β 2-rearrangement. For

by GS but only by 50% of the labs analyzing the PCR products by HD (GBS-4). Three samples were found to produce weak clonal signals only by HD analysis (ES-6, GBS-9 and DE-2). *TCRB* rearranged subclones being too small to be detected by SB analysis 5 may only be seen by the more sensitive PCR methodology. In B cell malignancies the detected rearrangements may also represent clonal or oligoclonal expansions of residual T cells.¹⁰⁵ In this case these weak clonal PCR products should not be regarded as evidence of a clonal T cell disorder. This stresses the importance of the interpretation of the PCR results in context with other diagnostic tests and the clinical picture of the patients.

10 Optimal PCR assessment of *TCRB* rearrangements is obtained by the combined use of HD and GS analysis. Sensitivity may differ between the two detection methods as a function of clonal PCR product size compared to the polyclonal size distribution: on the one hand HD analysis disperses the polyclonal background from the clonal products and on the other hand PCR products outside the main size range allow a more sensitive GS 15 detection. Also the risk of false-positive results is reduced in the combined use of HD and GS analysis. Furthermore, HD analysis allows detection of some additional atypical *TCRB* D β 1-D β 2 rearrangements that cannot be detected by GS analysis of the PCR product as no labeled primer is involved in amplification. However, GS analysis is in general the more informative method for samples with a high tumor load because the 20 exact size of the monoclonal PCR product is indicated, which may be used for monitoring purposes and differentially labeled J β primers provide additional information about J β gene usage.

Conclusion

25 In conclusion, the three-tube *TCRB* multiplex PCR system provides a new and convenient assay for clonality assessment in suspect T-cell proliferations with an unprecedentedly high clonality detection rate.

EXAMPLE 6: *TCRG* gene rearrangements**Background**

TCRG gene rearrangements have long been used for DNA PCR detection of lymphoid clonality and represent the "prototype" of restricted repertoire targets. It is a 5 preferential target for clonality analyses since it is rearranged at an early stage of T lymphoid development, probably just after *TCRD*,¹⁰⁶ in both *TCRαβ* and *TCRγδ* lineage precursors. It is rearranged in greater than 90% of T-ALL, T-LGL and T-PLL, in 50-75% of peripheral T-NHL and mycosis fungoïdes but not in true NK cell proliferations. It is also rearranged in a major part of B lineage ALLs, but much less so in B-NHL.^{1, 30, 73}

10 Unlike several other Ig/TCR loci, the complete genomic structure has been known for many years. It contains a limited number of *Vγ* and *Jγ* segments. Amplification of all major *Vγ*-*Jγ* combinations is possible with limited number of four *Vγ* and three *Jγ* primers.

The human *TCRG* locus on chromosome 7p14 contains 14 *Vγ* segments, only ten 15 of which have been shown to undergo rearrangement (Figure 8A). The *expressed* *Vγ* repertoire includes only six *Vγ* genes (*Vγ2*, *Vγ3*, *Vγ4*, *Vγ5*, *Vγ8* and *Vγ9*) but rearrangement also occurs with the ψ *Vγ7*, ψ *Vγ10*, ψ *Vγ11* segments.^{107, 108} Rearrangement of ψ *VγB* (also known as *Vγ12*)¹⁰⁷ is so exceptional that it is rarely used in diagnostic PCR strategies. Rearranging *Vγ* segments can be subdivided into those belonging to the *VγI* 20 family (*VγfI*: *Vγ2*, *Vγ3*, *Vγ4*, *Vγ5*, ψ *Vγ7* and *Vγ8*; overall homology > 90% and highest between *Vγ2* and *Vγ4* and between *Vγ3* and *Vγ5*) and the single member *Vγ9*, ψ *Vγ10*, ψ *Vγ11* families. The *TCRG* locus contains five *Jγ* segments: *Jγ1.1* (*JγP1*), *Jγ1.2* (*JγP*), *Jγ1.3* (*Jγ1*), *Jγ2.1* (*JγP2*), *Jγ2.3* (*Jγ2*), of which *Jγ1.3* and *Jγ2.3* are highly homologous, as are *Jγ1.1* and *Jγ2.1*.¹⁰⁹

25 Whilst the restricted *TCRG* germline repertoire facilitates PCR amplification, the limited junctional diversity of *TCRG* rearrangements complicates distinction between clonal and polyclonal PCR products. The *TCRG* locus does not contain D segments and demonstrates relatively limited nucleotide additions. *TCRG* V-J junctional length therefore varies by 20-30 bp, compared to approximately 60 bp for *IGH* and *TCRD*. The 30 capacity to distinguish clonal from polyclonal *TCRG* rearrangements depends on the complexity of the polyclonal repertoire. In general, minor clonal populations using frequent *Vγ*-*Jγ* rearrangements such as *VγfI*-*Jγ1.3/2.3* are at risk of being lost amidst the polyclonal repertoire, whereas rare combinations will be detected with greater sensitivity. However, occasional polyclonal T lymphocytes demonstrating rare *Vγ*-*Jγ* 35 rearrangements may be mistaken for a clonal rearrangement, due to absence of a

polyclonal background for that type of rearrangement. A further possible source of false positivity results from the presence of TCR $\gamma\delta$ expressing T lymphocytes demonstrating "canonical" *TCRG* rearrangements, which do not demonstrate N nucleotide additions. The most commonly recognized human canonical *TCRG* rearrangement involves the V γ 9-J γ 1.2 segments and occurs in approximately 1 % of blood T-lymphocytes.^{110,111} It is therefore extremely important to analyze *TCRG* PCR products using high resolution electrophoretic techniques or to separate PCR products on criteria other than purely on size, in order to reduce the risk of false positive results. It is also important to be aware of the profile of canonical rearrangements and the situations in which they most commonly occur. Canonical V γ 9-J γ 1.2 rearrangements, for example, are found predominantly in peripheral blood and increase in frequency with age, since they result from accumulation of TCR $\gamma\delta^+$ T-lymphocytes.¹⁹

Unlike *TCRD*, *TCRG* is not deleted in TCR $\alpha\beta$ lineage cells. Since *TCRG* rearrangements occur in both TCR $\alpha\beta$ and TCR $\gamma\delta$ lineage precursors, their identification cannot be used for determination of the type of T cell lineage. Similarly, *TCRG* rearrangements occur in 60% of B lineage ALLs,³⁰ implying that they can not be used for assessment of B vs. T cell lineage in immature proliferations. However, they occur much less frequently in mature B lymphoproliferative disorders, including the majority of B-NHL,¹ and might therefore be used, in combination with clinical and immunophenotypic data, to determine lineage involvement in mature lymphoproliferative disorders.

The limited germline repertoire allows determination of V γ and J γ segment utilization, either by Southern blot or PCR analysis. Identification of V γ and J γ usage is not of purely academic interest, since specific amplification is required for MRD analysis.¹¹²

We undertook to develop a minimal number of multiplex *TCRG* strategies which would maintain optimal sensitivity and informativity, minimize the risk of false positive results and allow simple V γ and J γ identification, including by HD analysis or monofluorescent GS strategies. We chose to include V γ primers detecting all rearranging segments other than ψ V γ B (ψ V γ 12), given its rarity. In order to reduce the risk of falsely identifying canonical rearrangements as clonal products, we excluded the J γ 1.2 primer, since it is rarely involved in lymphoid neoplasms and is usually, although not always, associated with a *TCRG* rearrangement on the other allele.¹¹³

Primer design

We initially developed 3V γ and 2 J γ primers, to be used in two multiplex reactions, as follows: one tube with J γ 1.3/2.3 with V γ 9 specific (160-190 bp), V γ fI consensus (200-230 bp) and V γ 10/11 consensus (220-250 bp) and a second tube with J γ 1.1/2.1 with V γ 9

5 specific (190-220 bp), V γ fI consensus (230-260 bp) and V γ 10/11 consensus (250-280bp). V γ usage was to be identified by PCR product size by HD analysis. No distinction between J γ 1.3 and J γ 2.3 or J γ 1.1 and J γ 2.1 was attempted.

Results of initial testing phase

10 While all V γ -J γ combinations gave the expected profiles on single PCR amplification, multiplex amplification led to competition of larger PCR products, with preferential amplification of smaller fragments, and failure to detect some V γ fI and V γ 10/11 rearrangements. This was further complicated by significant primer dimer formation between the V γ 10/11 consensus and the V γ fI primers. Competition between 15 differently sized fragments and primer dimer formation both led to unsatisfactory sensitivity and informativity and this strategy was therefore abandoned.

20 We reasoned that competition would be minimized by separating the most frequently used V γ primers (V γ fI and V γ 9) and combining them with V γ 10 and V γ 11 specific primers, respectively. The latter rearrangements are rarely used and therefore minimize competition for the predominant repertoires. The V γ 10/11 consensus primer was therefore replaced by two specific V γ primers which generated smaller PCR products (Figure 8B). By mixing J γ 1.3/2.3 and J γ 1.1/2.1 it was possible to maintain a two-tube multiplex which allows approximate identification on the basis of product size of V γ usage by HD analysis and of both J γ and V γ usage by GS analysis.

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The approved set of multiplex TCRG PCR tubes with four V γ and two J γ primers includes (Figure 8B):

Tube A: V γ fI + V γ 10 + J γ 1.1/2.1+ J γ 1.3/2.3

Tube B: V γ 9 + V γ 11 + J γ 1.1/2.1+ J γ 1.3/2.3

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The position and the sequence of the primers are shown in Figure 8B. These primers gave satisfactory amplification in both single and multiplex PCR formats and allowed detection of virtually all known V γ -J γ combinations. The competition of larger PCR fragments was no longer seen, although it cannot be excluded that some competition of 35 V γ 9 or V γ fI rearrangements may occur if these are present in a minority population.

the polyclonal repertoire and the position of the clonal rearrangement relative to the polyclonal Gaussian peak.¹⁴ Interpretation of $\psi V\gamma 11$ rearrangements can be difficult, since the normal repertoire is extremely restricted and since these primitive rearrangements are often present in subclones.

5 Since the $V\gamma 4$ segment is approximately 40 bp longer than the other $V\gamma f1$ members and $V\gamma 4$ rearrangements are relatively common in both physiological and pathological lymphoid cells, the polyclonal repertoire can be skewed towards larger sized fragments, 10 and clonal $V\gamma 4$ - $J\gamma 1.3/2.3$ rearrangements could theoretically be mistaken for $V\gamma f1$ - $J\gamma 1.1/2.1$ rearrangements. The proximity of the different repertoires also makes $V\gamma$ and $J\gamma$ identification much more reliable if differently labeled $J\gamma$ primers are used. For example, the use of a TET-labeled $J\gamma 1.1/2.1$ and a FAM labeled $J\gamma 1.3/2.3$ was tested in a single center and was shown to give satisfactory results (Figure 13B). It is, however, 15 possible to estimate $V\gamma$ and $J\gamma$ usage following GS analysis on the basis of size alone (Figure 8C and D).

Results of general testing phase

Given the limited germline $TCRG$ repertoire and the restricted junctional 20 diversity, reactive T lymphocytes which have undergone $TCRG$ rearrangements using a single $V\gamma$ and $J\gamma$ segment with variable CDR3 sequences which are of uniform length, will migrate as an apparent clonal population by GS analysis. HD formation will disperse these rearrangements more easily and will therefore prevent their erroneous interpretation as evidence of lymphoid clonality. In contrast, GS analysis provides 25 improved resolution and sensitivity compared to HD analysis. For these reasons, optimal assessment of $TCRG$ rearrangements requires both HD and GS analysis. If this is not possible, HD analysis alone is probably preferable, since it might be associated with a risk of false-negative results, whereas GS analysis alone will increase the risk of false-positive results.

30 Of the 18 $TCRG$ rearrangements detected by Southern blotting in the 90 cases, 16 were also detected by PCR. The minor $V\gamma f1$ - $J\gamma 1.3/2.3$ rearrangement detected by Southern in the NL-1 oligoclonal case, was only detected by PCR in a proportion of laboratories performing GS analysis. A major $V\gamma 9$ - $J\gamma 1.3/2.3$ rearrangement detected in GBS-6 was found to be polyclonal by both HD and GS in all laboratories and, as such, 35 probably represents a false-negative result.

Comparison of allele identification showed that, for all alleles identified by Southern blotting, PCR $V\gamma$ and $J\gamma$ identification on the basis of size gave concordant results. Seven rearrangements were detected by Southern blotting but precise allele identification was not possible. Six of these were due to $J\gamma 1.1/2.1$ usage, suggesting that 5 PCR allows preferential detection of this type of rearrangement.

Seventy two samples were considered to be polyclonal by Southern. Sixteen (22%) of these demonstrated a total of 24 rearrangements by *TCRG* PCR. Of these, 13 (81%) were B lymphoid proliferations. Sixteen of the 24 clonal rearrangements were minor, with 15 only being detected by GS in the majority of laboratories. It is worth noting that, 10 of these minor rearrangements, nine (39%) involved the $\psi V\gamma 10$ segment and eight (33%) $V\gamma 9$. $\psi V\gamma 11$ rearrangements were not detected. No $\psi V\gamma 10$ rearrangements were detected by Southern blot analysis. PCR therefore allowed more sensitive detection of minor 15 clonal $\psi V\gamma 10$ rearrangements, particularly by GS analysis. It is likely that these rearrangements represent residual, predominantly $TCR\alpha\beta$ lineage, T lymphocytes with a restricted repertoire, which may or may not be related to the underlying B lymphoid malignancy. These minor peaks should obviously not be interpreted as evidence of a 20 clonal T cell disorder. They emphasize the importance of understanding the nature of *TCRG* rearrangements before using this locus as a PCR target in the lymphoid clonality diagnostic setting. Consequently, it is also extremely important to interpret *TCRG* gene results within their clinical context.

Conclusion

In conclusion, the two *TCRG* multiplex tubes allow detection of the vast majority of 25 clonal *TCRG* rearrangements. The potential risk of false positive results due to over-interpretation of minor clonal peaks can be minimized by the combined use of heteroduplex analysis and GeneScanning and by interpreting results within their clinical context, particularly when the apparent clonality involved the $\psi V\gamma 10$ and $\psi V\gamma 11$ segments. The relative merits of *TCRG* compared to *TCRB* analysis for the detection of 30 clonal T lymphoproliferative disorders should be studied prospectively. They are likely to represent complementary strategies.

Background

The human *TCRD* gene locus is located on chromosome 14q11.2 between the $V\alpha$ and $J\alpha$ gene segments. The major part of the *TCRD* locus ($D\delta$ - $J\delta$ - $C\delta$) is flanked by 5 *TCRD*-deleting elements, $\psi J\alpha$ and δ REC such that rearrangement of the deleting elements to each other or rearrangement of $V\alpha$ to $J\alpha$ gene segments causes deletion of the intermediate *TCRD* gene locus (Figure 9A). The germline encoded *TCRD* locus consists of 8 $V\delta$, 4 $J\delta$, and 3 $D\delta$ gene segments, of which at least five of the eight $V\delta$ gene segments can also rearrange to $J\alpha$ gene segments.¹¹⁵ Other $V\alpha$ gene segments may also 10 be utilized in *TCRD* gene rearrangements in rare cases. The WHO-IUIS nomenclature¹¹⁶ for TCR gene segments uses a different numbering system for those V genes used mainly or exclusively in TCR δ chains from those which can be used in either TCR α or TCR δ chains. Thus *TCRDV101S1* ($V\delta1$), *TCRDV102S1* ($V\delta2$) and *TCRDV103S1* ($V\delta3$) are used almost exclusively in *TCRD* rearrangements, whereas *TCRADV6S1* ($V\delta4$), 15 *TCRADV21S1* ($V\delta5$) and *TCRADV17S1* ($V\delta6$) can be used in either TCR δ or α chains. *TCRADV28S1* ($V\delta7$) and *TCRADV14S1* ($V\delta8$) are used extremely rarely in *TCRD* rearrangements.

The germline-encoded repertoire of the TCR $\gamma\delta^+$ T cells is small compared to the TCR $\alpha\beta^+$ T cells and the combinatorial repertoire is even more limited due to preferential 20 recombination in peripheral blood and thymocyte TCR $\gamma\delta^+$ T cells. At birth, the repertoire of cord blood TCR $\gamma\delta^+$ T cells is broad, with no apparent restriction or preferred expression of particular $V\gamma$ / $V\delta$ combinations. During childhood, however, the peripheral blood TCR $\gamma\delta^+$ T cell repertoire is strikingly shaped so that $V\gamma9/V\delta2$ cells clearly dominate 25 in adults.¹¹⁷ Studies have shown that $V\delta1$ and $V\delta2$ repertoires become restricted with age leading to the appearance of oligoclonal $V\delta1^+$ and $V\delta2^+$ cells in blood and intestine.¹¹⁸ TCR $\gamma\delta^+$ T cells are evenly distributed throughout human lymphoid tissues but there is preferential expression of particular $V\delta$ segments in specified anatomical localizations. Notably, most intraepithelial TCR $\gamma\delta$ T cells occurring in the small intestine and in the 30 colon express $V\delta1$. Similarly, $V\delta1$ is expressed by normal spleen TCR $\gamma\delta^+$ T cells, but TCR $\gamma\delta^+$ T cells in the skin express the $V\delta2$ gene.

Although the small number of V, D and J gene segments available for recombination limits the potential combinatorial diversity, the CDR3 or junctional diversity is extensive due to the addition of N regions, P regions and random deletion of nucleotides by recombinases. This diversity is also extended by the recombination of up 35 to three $D\delta$ segments and therefore up to four N-regions within the rearranged *TCRD*

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locus. This limited germline diversity encoded at the *TCRD* locus in conjunction with extensive junctional diversity results in a useful target for PCR analysis and *TCRD* recombination events have been used most extensively as clonal markers in both T and B cell acute lymphoblastic leukemia (ALL).^{119, 120} The *TCRD* locus is the first of all TCR loci to rearrange during T cell ontogeny. The first event is a D δ 2-D δ 3 rearrangement, followed by a V δ 2-(D δ 1-D δ 2)-D δ 3 rearrangement, and finally V δ -D δ -J δ rearrangement. Immature rearrangements (V δ 2-D δ 3 or D δ 2-D δ 3) occur in 70% of precursor B-ALL (and are therefore non lineage restricted)³⁰ while there is a predominance of mature rearrangements comprising incomplete D δ 2-J δ 1 and complete V δ 1, V δ 2, V δ 3 to J δ 1 found in T-ALL.^{23, 121} Thus specific primer sets can be used to identify different types of complete and incomplete rearrangements corresponding to different types of ALL.¹²²

TCR γ δ ⁺ T-ALL form a relatively small subgroup of ALL, representing 10-15% of T-ALL but still only constitute 2% of all ALL. V δ 1-J δ 1 rearrangements predominate in TCR γ δ ⁺ T ALL; interestingly V δ 1 is never found in combination with J δ segments other than J δ 1.^{15, 20} Other recombinations occur in less than 25% of alleles. Furthermore, V δ 1-J δ 1-C δ chains are almost always disulfide linked to either V γ I or V γ II gene families recombined to J γ 2.3-Cy2. Such gene usage is consistent with the immature thymic origin of these leukemic cells.

Most T cell lymphomas express TCR α β while the minority express TCR γ δ and comprise of several distinct entities. Peripheral T cell lymphomas (PTCL) expressing TCR γ δ comprise 8-13% of all PTCL and V δ 1-J δ 1 as well as other V δ to J δ 1 recombinations have been documented.^{123, 124} Hepatosplenic γ δ T-cell lymphoma is derived from splenic TCR γ δ T cells which normally express V δ 1. It is an uncommon entity that exhibits distinctive clinicopathologic features and gene usage analysis has indicated clonal V δ 1-J δ 1 rearrangements associated with these lymphomas.¹²⁵ Furthermore, the rare type of cutaneous TCR γ δ ⁺ T cell lymphomas express V δ 2 and therefore appear to represent a clonal expansion of TCR γ δ ⁺ T cells which normally reside in the skin.¹²⁶ Other clonal TCR γ δ proliferations include CD3 $^{+}$ TCR γ δ ⁺ large granular lymphocyte (LGL) proliferations which comprise about 5% of all CD3 $^{+}$ LGL and often show V δ 1-J δ 1 rearrangements.¹²⁷

The development of monoclonal antibodies towards framework regions of TCR γ δ and more recently to specific V δ gene segments has helped identify TCR γ δ ⁺ T cell populations by flow cytometric analysis,¹⁵ but PCR clonality studies are still required to identify whether these populations represent clonal or polyclonal expansions.¹²⁸

The *TCRD* gene segments, consisting of eight *V δ* , four *J δ* and three *D δ* gene segments, show little or no homology to each other and so segment-specific primers were designed which would not cross-anneal with other gene segments. Usage of *V δ 7* and *V δ 8* gene segments was considered too rare to justify inclusion of primers for these segments and so, following the general guidelines according to the invention for primer design, a total of 16 primers were designed: 6 *V δ* , 4 *J δ* and 5' and 3' of the 3 *D δ* gene segments (Figure 9B). All primers were designed for multiplex together in any combination, but originally it was planned to have one tube (A) with all *V* and all *J* primers which would amplify all the complete *V(D)J* rearrangements and a second tube (B) with *V δ 2*, *D δ 2-5'*, *D δ 3-3'* and *J δ 1* primers to amplify the major partial rearrangements (*V δ 2-D δ 3*, *D δ 2-D δ 3* and *D δ 2-J δ 1*). Together these tubes should amplify 95% of known rearrangements. The other primers (*D δ 1-5'*, *D δ 3-5'*, *D δ 1-3'* and *D δ 2-3'*) could be used to amplify other *D δ -J δ* , *V δ -D δ* or *D δ -D δ* rearrangements, but were always intended to be optional.

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Results of initial testing phase

All primer pair combinations were tested using polyclonal DNA (tonsil and MNC). Most gave products of the expected size, but some (*D δ 1-5'*, *D δ 1-3'* and *D δ 2-3'*) gave no visible product in combination with any other primer. Rearrangements involving these primer regions are likely to be extremely rare and so these, and *D δ 3-5'*, were excluded from subsequent testing. Clonal cases for the six main rearrangements (*V δ 1-J δ 1*, *V δ 2-J δ 1*, *V δ 3-J δ 1*, *D δ 2-D δ 3*, *V δ 2-D δ 3* and *D δ 2-J δ 1*) were tested initially in monoplex PCR and then in multiplex tubes A and B (see above). Serial dilutions of clonal DNA in polyclonal DNA (tonsil or MNC) showed detection sensitivities of at least 5% in all cases. However, in clonal cases with biallelic rearrangements, which were clearly detected in single PCR reactions, the second, usually larger, allele often failed to amplify on multiplexing. In addition, it was found, using a different set of clonal cases that several of the *V δ 2-J δ 1* rearrangements failed to amplify. A polymorphic site was subsequently identified at the position of the original *V δ 2* primer;¹²⁹ the frequency of this polymorphism in the general population unknown, and so this primer was redesigned to a new region of the *V δ 2* gene segment, retested and found to amplify all cases. The problem with the failure to amplify the second allele was overcome by increasing the $MgCl_2$ concentration from 1.5 mM to 2.0 mM.

We also tested the possibility of combining the two tubes into a single multiplex reaction. Twelve clonal cases were tested, which had a total of 21 gene rearrangements

between them. A single multiplex tube containing 12 primers (6 V δ , 4 J δ , D δ 2-5' and D δ 3-3') was used with ABI Gold buffer and 2.0 mM MgCl₂ to amplify all the cases. All gene rearrangements were indeed detected with a sensitivity of 0.5-10% by HD analysis when diluted in polyclonal MNC DNA (Table 7). The only problem with combining all 5 *TCRD* primers in a single tube was the appearance of a nonspecific band at about 90 bp in all amplifications, which was not present when the two separate multiplex tubes were used. Since the band was outside the size range of the *TCRD* products and did not interfere with interpretation, it was not considered to be a problem.

10 Results of general testing phase

The testing of the 90 Southern blot-defined samples in ten laboratories raised some general issues about the *TCRD* protocol:

Interpretation of some GS results was difficult. Because of the large size range of products for the *TCRD* locus, there is no classical Gaussian distribution for polyclonal 15 samples (see Figure 9C) and this, coupled with the low usage of *TCRD* in many samples meant that in some cases it was hard to determine whether a sample was polyclonal or clonal. The same problem did not arise with HD analysis and so the recommendation is that GS should only be used for *TCRD* with extreme care and awareness of the potential 20 problems.

25 The 90 bp nonspecific band was quite intense in some laboratories, but less so in others. It appeared to be weaker when using Buffer II rather than Gold buffer (confirmed by subsequent testing) and is also sensitive to MgCl₂ concentration, becoming more intense as MgCl₂ concentration increases. This product has now been sequenced and found to be an unrelated gene utilizing the D δ 2 and J δ 3 primers.

30 The results of the general testing of the 90 Southern blot defined samples showed that the overall concordance of all the PCR groups doing the testing was very high (95%). Of the 90 cases, six were Southern blot positive for *TCRD* clonal rearrangements, five of which were found to be clonal by PCR. The remaining case (DE-10, a T-ALL with high tumor load) was found to be polyclonal by all labs. Of the 84 Southern blot negative 35 cases, 75 were found to be polyclonal by PCR, four were found to be clonal and the remaining five cases showed discordance between the GS and HD results. Of the clonal cases, two (DE-2 and GBS-9) were T-rich B-NHLs with presumably low tumor load and so the results may reflect the increased sensitivity of PCR over Southern blotting. The other two clonal cases (GBS-15 and ES-7) had high tumor load. Of the five cases, which 40 showed discrepancy between the GS and HD results, one (NL-1) was a difficult

ongenital case, which caused problems for several other too. The remaining four were found to be polyclonal by HD and clonal by GS. In three of the cases (NL-13, NL-15 and NL-18) this may reflect the greater sensitivity of GS over HD analysis, but the remaining case (PT-1, a reactive lymph node) may be attributed to "pseudyclonality" on 5 GS analysis because of the limited repertoire of *TCRD* usage in some samples.

Conclusion

In conclusion, the recommended protocol for detection of *TCRD* gene rearrangements is a single tube assay containing 12 primers for detection of all major 10 *V* δ (*D*)*J* δ , *V* δ -*D* δ , *D* δ -*D* δ and *D* δ -*J* δ rearrangements using Buffer II and 2.0 mM MgCl₂ to ensure maximum specificity and detection. The preferred analysis method is HD, but GS may be used with care if consideration is given to the problems of pseudyclonality caused by the limited usage of *TCRD* in some samples. However, the use of multi-color 15 GeneScanning (see Figure 13C) can be helpful in rapid recognition of the different types of complete and incomplete *TCRD* gene rearrangements in the different types of ALL. With these limitations in mind, *TCRD* can nevertheless be a valuable target for the more immature T-cell leukemias as well as TCR γ δ ⁺ T-cell proliferations.

EXAMPLE 8. t(11;14) with *BCL1*- *IGH* rearrangementBackground

The t(11;14)(q13;q32) is characteristic for mantle cell lymphoma (MCL) because this cytogenetic reciprocal translocation was observed in 60-70% of MCL cases and only 5 sporadically in other B-cell NHL.¹³⁰ The breakpoint region was originally cloned by Tsujimoto et al (1983) and referred to as the *BCL1*-region.¹³¹ However in only few cases with a cytogenetic t(11;14) a genomic breakpoint in the *BCL1*-region was identified. Using fiber and interphase FISH with probes covering the approximately 750 kb 11q13-*BCL1* region, in almost all MCL (33 out of 34) a breakpoint was observed and all 10 breakpoints were confined to a region of 360 kb 5' of the cyclin D1 gene.^{132, 133} In nearly half of MCL cases (41%) the breakpoints were clustered within an 85 bp region that was referred to as the major translocation cluster region, *BCL1*-MTC.^{130, 134, 135} In most if not all cases of MCL the break at the *IGH* locus located at 14q32 involves the JH genes 15 juxtaposing the *IGH*-Eμ enhancer to chromosome 11q13 sequences and consequently resulting in transcriptional activation of the cyclin D1 gene.¹³⁶ Cyclin D1 together with CDK4 phosphorylates (and inactivates) pRB and allows for progression through the G1 phase of the cell cycle. Because cyclin D1 is silent in B-lymphocytes and B-cell NHL other than MCL, and the presence of this translocation correlates well with cyclin D1 expression, this gene is considered to be the biological relevant target in MCL.¹³⁶ Both 20 expression of cyclin D1 and/or the presence of t(11;14)(q13;q32) is used as an additional tool in the differential diagnosis of NHL.² The gold standard detection strategy for the presence of the t(11;14) that will identify almost all breakpoints is interphase FISH using breakpoint-flanking probes in fresh or frozen material¹³³ as well as in archival specimens.¹³⁷ However, a PCR based detection strategy for the t(11;14) might be useful 25 for e.g. residual disease monitoring. Many groups have developed PCR based assays to detect the *BCL1*/JH-breakpoints, in general using a consensus JH-primer in combination with primers in the *BCL1*-MTC region that were all located in a region of 392 bp.^{54, 55} Breaks within the *BCL1*-MTC region can occur upto 2 kb downstream of the MTC region, but the majority of breakpoints are tightly clustered within an 85 bp segment, 30 immediately downstream of the reported most 3'-primer ("primer B" in ^{54, 134}). Because breaks in this *BCL1*-MTC-region account for only part of the breakpoints in the 11q13-*BCL1* region in MCL cases (41%), the PCR based strategy for t(11;14) seriously impairs the diagnostic capability with an high rate of false-negative results as compared to FISH.

The t(11;14)(q13;q34) has also been reported to be observed in CLL and other proliferative diseases such as multiple myeloma (20%), SLVL (30%), B-PLL (33%) and B-CLL (8%).^{130, 138, 139} One reason for the presence of the t(11;14) in B-CLL in some studies might be due to the incorrect classification of B-CLL.¹³⁰ In myeloma the breakpoints are quite different from those in MCL because (i) the frequency is much lower; (ii) most breaks involve switch-class recombination sites; and (iii) although all tested cases are located in the same 360 kb *BCL1*-region there seems to be no preferential clustering within the *BCL1*-MTC region. On the other hand, in all cases with a break the cyclin D1 gene is activated. Of note, in a subgroup of multiple myelomas with a *IGH*-switch-break 10 myeloma, an additional region in the 11q13-*BCL1* region, is involved.¹³⁸

Primer design

Based on the location of the reported most-far 5'-breakpoint and available 15 nucleotide sequences from the *BCL1*-MTC region (GenBank accession number S77049), we designed a single *BCL1* primer (5'-GGATAAAGGCGAGGAGCATAA-3') in the 472-bp region 5' of this breakpoint by using the primer design program Oligo6.2 relative to the consensus JH primer.

Results of initial testing phase

Using the consensus JH-primer in combination with the single *BCL1*-MTC-primer 20 on a small series of MCL (n=5) previously identified as positive with an in-house *BCL1*/JH-PCR using a similar consensus JH18-primer (18 nt) and 5'-GCACGTCTGGATGCACCGC-3' as *BCL1*-MTC-primer, we initially compared both assays in parallel. In contrast to the analysis of Ig/TCR gene rearrangements via GS 25 and/or HD analysis, the *BCL1*-JH PCR products (as for *BCL2*-JH products) are identified via agarose gel electrophoresis using ethidium bromide staining only. The results on the five positive and two negative samples were identical except that the PCR products were significantly weaker. To evaluate whether we could increase the sensitivity of the PCR, we determined the effect of different concentrations of MgCl₂ and primers, and different 30 temperatures in a Stratagene-Robocycler PCR-machine (all other PCR were done on ABI-480 or ABI-9700). Most intriguing was the variation due to small changes in MgCl₂ concentration. At 2.0 mM a weak nonspecific product of 550 bp became apparent whereas at 2.5 mM and higher this nonspecific product was very prominent in all DNAs including non-template DNA controls. At lower concentrations (less than 1.5 mM) no 35 nonspecific fragments were observed but the expected specific products were very weak.

Hybridizations with a *BCL1*-MTC-internal oligo-probe (5'-ACCGAATATGCAGTGCAGC-3') did not show hybridization to this 550 bp product. PCRs with each of the primers separately revealed that the 550 bp product could be generated by using the JH-consensus primer only. In some MCL cases, in addition to the PCR-products ranging 5 from 150-350 bp (Figure 10B), larger specific PCR-products might be apparent due to annealing of the consensus JH-primer to downstream JH5 and JH6 segments as described for *BCL2*/JH.¹⁴⁰ From the initial testing phase the most optimal PCR-conditions for the *BCL1*-MTC/JH-PCR were: annealing temperature of 60°C, 2.0 mM MgCl₂ and 10 pmol of each primer (for 35 PCR-cycles in the ABI 9700).

10 To evaluate the specificity of the PCR on a larger series of cases, the *BCL1*-MTC/JH-PCR was performed in three laboratories on DNA from in total 25 cases MCL that were all previously identified as positive with in-house *BCL1*/JH-PCR, and from 18 negative controls. None of the negative cases revealed a PCR-product whereas 22 of 25 positive cases showed products of the expected size. In the three cases that did not reveal 15 a product on agarose-gel, a product was detected with GS suggesting that the sensitivity is lower when compared to in-house PCR.

10 The sensitivity of the PCR was evaluated by amplifying DNA dilutions of a MCL in normal tonsillar DNA. A sensitivity between 10⁻³ and 10⁻⁴ was observed on agarose gel using the developed PCR-primers. An in-house PCR performed in parallel on the same 20 samples was at least 10x more sensitive. Hybridizations with the in-house *BCL1*-MTC-oligo-probe revealed a 10-100x higher sensitivity of both PCRs. Dilutions with DNA of an established cell line JVM2 (available through DSMZ; <http://www.dsmz.de>) with an *BCL1*-MTC/JH4-breakpoint⁵³ is used as our standard positive control. As a negative 25 control normal tonsillar tissue or peripheral blood cells might be used, but almost any non-MCL B-cell NHL should be suitable because of the very low frequency of this aberration.¹³⁰

Results of general testing phase

30 To evaluate inter-laboratory variations for the detection of breakpoints at the *BCL1*-MTC region, ten groups participated in the analysis of DNA from a series of 90 histologically defined malignant and reactive lymphoproliferations using the *BCL1*-MTC/JH-PCR protocol. All cases were defined for their status at the Ig and TCR loci using Southern hybridization techniques. Of the 90 cases, seven were histologically characterized as MCL. All seven MCL cases were shown to have a clonal *IGH* 35 rearrangement by Southern hybridization. Assessment of rearrangements within the

not performed in all cases. In six of the seven MCL cases the PCR-product was identified in all ten laboratories. In MCL case NL-15 in six of the laboratories the expected 1.8 kb PCR product was identified. This particular case carries an exceptional breakpoint with an uncommon large PCR-product (normally ranging from 150 to 350 bp) and represents the 3'-most-far detectable *BCL1*-MTC-breakpoint to our knowledge. In two of six labs the PCR product was observed but initially considered as nonspecific because of its uncommon size. In ES-4, characterized histologically as MCL in none of the ten labs a PCR-product could be detected suggesting that this case carries a breakpoint outside the *BCL1*-MTC. It should be stressed that the MCL cases submitted to this series for the general testing phase were selected and thus are expected to carry breaks at the *BCL1*-MTC region at an higher incidence than normal. Importantly, except for one single case (FR-1), in all 83 other non-MCL cases including 16 cases that were histologically characterized as B-CLL, no *BCL1*- MTC/JH-PCR product was detected in any laboratory.

In case FR-1 histologically characterized as B-CLL, in three of the ten labs a product was identified indicating that the number of cells with this break is low. The *IGH* status determined by Southern blot analysis revealed that this sample was composed of 90% clonal B-cells in good agreement with the histological examination. PCR-based B-cell clonality analyses for *IGH* and *IGK* (sensitivity of approximately 1%) revealed a single clone and Southern blot analysis for *IGK* showed a single major *IGK* rearrangement only. In addition, Northern blot analysis for expression of cyclin D1 did not show overexpression. All these data suggested that the very small number (less than 1%) t(11;14)-positive cells represent either (i) a subclone derived from the B-CLL, (ii) an independent second B-malignancy or (iii) normal B-cells as described for t(14;18)-positive B-cells in normal individuals.⁴⁰ However, with the available data of this patient at present we can not discriminate between these three alternatives. In summary, the analysis by the ten laboratories illustrates the high specificity of the *BCL1*- MTC/JH-PCR strategy.

To evaluate the presence of possible false-negative cases due to the relative low sensitivity of the PCR, in one laboratory the previously described in-house PCR (with about 10-fold higher sensitivity) was performed on DNA of all 90 cases and the PCR products of both assays were also hybridized with an internal-*BCL1*-MTC oligo-probe that increases the sensitivity another 10-100-fold. This analysis revealed no PCR products in other cases.

Conclusion

We conclude that also the sensitivity of the *BCL1*-MTC/JH PCR (between 10⁻³ and 10⁻⁴) is sufficiently high for the detection of the *BCL1*-MTC /JH-breakpoint in diagnostic material. The results of this approach are very encouraging and suggest that the 5 definition of common approaches and reaction conditions can minimize erroneous results. However, it should be remembered that maximally about 50% of the t(11;14) breakpoints in MCL will be detected and that for diagnosis additional detection tools are recommended.

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EXAMPLE 9. t(14;18) with *BCL2-IGH* rearrangement

Background

The t(14;18) is one of the best characterized recurrent cytogenetic abnormalities in peripheral B cell lymphoproliferative disease.¹⁴¹ It is detectable in up to 90% of 15 follicular lymphomas and 20% of large cell B-cell lymphomas depending upon the diagnostic test used.¹⁴² As a consequence of the translocation the *BCL2* gene from 18q32 is placed under the control of the strong enhancers of the *IGH* locus resulting in deregulation of its normal pattern of expression.^{143, 144} *BCL2* is located on the outer mitochondrial membrane and its normal function is to antagonize apoptosis and when 20 deregulated it is intimately involved in the pathogenesis of the tumor.¹⁴⁵⁻¹⁴⁸ As a consequence of this role in pathogenesis the t(14;18) provides an ideal target for both diagnosis and molecular monitoring of residual disease.

The *IGH* locus is located at 14q32.3 with the V_H regions lying telomeric and the D_H, J_H and constant regions placed more centromeric. The transcriptional orientation is 25 from telomere to centromere with enhancers located 5' of the V regions and between each of the constant regions. The most common form of the translocation involves the process of VDJ recombination and one of the six germline J_H regions is closely opposed to *BCL2*. Most PCR based detection strategies have utilized a consensus J_H primer that will detect the majority of translocations.^{149, 150} In contrast to the *IGH* locus, the pattern of breaks in 30 *BCL2* is more complicated. *BCL2* is located on chromosome 18q21 and is orientated 5' to 3' from centromere to telomere. The majority of breakpoints fall within the 150 bp MBR located in the 3' untranslated region of exon 3.¹⁵¹ As a consequence of the translocation, the S μ enhancer located 3' of the J_H regions is placed in close proximity to the *BCL2* gene leading to its deregulation. As more translocations have been investigated it has become 35 apparent that there are a number of other breakpoint regions which must be taken into

is a further breakpoint region, the 3' MBR subcluster, encompassing a region of 3.8 kb.¹⁵² The mcr is located 20 kb 3' of the MBR and covers a region of 500 bp.¹⁵³ However, though analogous to the MBR, the mcr is more extensive than was initially envisaged and a region 10 kb upstream of the mcr, the 5' mcr subcluster, has been described.^{154, 155} In addition to these classical breakpoints a number of variant translocations are described where the breaks occur 5' of *BCL2*.¹⁵⁶ These are, however, rare and thus can not be taken into account using a PCR based detection strategy.

There is no single gold standard detection strategy for the t(14;18) and a combination of cytogenetics and Southern blotting have been generally used.^{157, 158} Interphase FISH detection strategies offer an applicable alternative that have the potential to pick up more translocations.¹⁵⁹ In contrast DNA based fiber FISH has been very informative for defining variant translocations but is unsuitable for routine application.¹⁶⁰ For molecular diagnostic laboratories PCR based detection strategies offer rapid results, are generally applicable and can be used for residual disease monitoring. However, the primers commonly used have been derived on an *ad hoc* basis and have not been designed to take into account recent information on the molecular anatomy of the breakpoints. As a consequence when compared to gold standard approaches, PCR based techniques only detect up to 60% of translocations which seriously impairs the diagnostic capability of PCR. Compounding this high percentage of false negative results is the problem of false positive results arising from contamination from other samples and previously amplified PCR products.

Primer design

We initially evaluated a two tube multiplex system, one tube designed to detect breakpoints within the MBR and a second tube used to identify breakpoints outside this region. The MBR strategy contained three primers MBR1, MBR2 and the consensus JH primer. The second multiplex reaction contained five primers, MCR1, MCR2, 5'mcr, 3' MBR1 and the consensus JH (Figure 11A) and was designed to detect breakpoints within the mcr, 5'mcr and 3' MBR regions.

Results of initial testing phase

The evaluation of these primers was performed in three laboratories on DNA derived from a total of 124 cases of follicular lymphoma known to carry a t(14;18). 109 cases (88%) were identified with an *BCL2-IGH* fusion, 83/124 (67%) were positive using

the MBR multiplex and 26/124 (21%) were positive using the non-MBR multiplex strategy. In 15/124 (12%) cases there was no amplifiable PCR product. Further examination of the cases identified with the non-MBR multiplex showed that 11 (9%) had a breakpoint within the mcr, five cases (4%) within the 5'mcr and 10/124 (8%) within 5 the 3'MBR.

To further investigate the value of this set of primers for the detection of breakpoints within the 5'mcr and 3'MBR sub-cluster regions a series of 32 cases of t(14;18) positive follicular lymphomas known to be germline at the MBR and mcr by Southern hybridization were analyzed in one laboratory. Five of the cases had 10 breakpoints within the 5'mcr (260-490bp) and were amplified using both the 5'mcr primer in isolation and with the multiplex reaction. None of the remainder of cases showed a positive result. Of the series of 32 cases, nine were already known to have breakpoints within the 3'MBR region and the multiplex approach was able to detect 5/9 of these cases.

15 In order to improve the sensitivity of the assay within this region we designed three further primers that spanned the 3'MBR sub-cluster region; 3'MBR2, 3'MBR3 and 3'MBR4 and combined them with 3'MBR1 and the consensus JH in an additional multiplex reaction; 3'MBR multiplex (Figure 11). This new approach confirmed that eight of the 32 cases were positive but missed the ninth case. The primers were then 20 used individually and in this experiment 11 of the 32 cases were positive. The breakpoints were distributed as follows; 2/11 cases had a breakpoint present between primer 3'MBR1 and 3'MBR2, 3/11 cases between primers 3'MBR2 and 3'MBR3, 2/11 cases between primers 3'MBR3 and 3'MBR4 and the remaining four cases amplified using primer 3'MBR4 and were distributed 200-1000bp 3' of this primer. In this series of 25 cases there were three false negative results using the 3'MBR multiplex. One of the cases was a true false negative where the break occurred in the middle of the 3'MBR, in proximity to an Alu repeat sequence. The translocation was detected using the 3'MBR3 primer when used in isolation and a product of 450 bp was generated suggesting a reduced sensitivity of the multiplex. The remaining two false negative cases generated 30 products larger than 1000bp with the 3'MBR4 primer, placing them in the far 3'MBR not fully covered by this approach. Further improvement in the sensitivity of the 3'MBR assay has been achieved following the general testing phase of this study. Substituting primer 3'MBR3 with a new downstream primer 5'-GGTGACAGAGCAAAACATGAACA- 3' (see Figure 11A) significantly improved both the sensitivity and specificity of the 35 3'MBR assay.

Based on this, the 5 tube multiplex was incorporated into our diagnostic strategy. Analysis of the Southern blot defined cases was therefore carried out using the three tube multiplex system presented in Figure 11A.

5 Results of general testing phase

Inter-laboratory variations feature significantly in diagnostic PCR strategies. To evaluate this, 11 groups participated in an extensive external quality control exercise. DNA was extracted from a series of 90 histologically defined malignant and reactive lymphoproliferations were analyzed using the t(14;18) multiplex protocol (Figure 11B, C, 10 and D). All cases were defined for their status at the Ig and TCR loci using Southern hybridization techniques. Karyotypic confirmation of the t(14;18) was not available on this series. We therefore adopted an approach requiring greater than 70% concordance between members of the network for acceptance of the t(14;18). Of the 90 cases, 11 were characterized histologically as follicular lymphoma. All 11 cases were shown to have a 15 clonal *IGH* rearrangement by Southern hybridization. Assessment of rearrangements within the *BCL2* gene was also performed by Southern hybridization using specific probes to the MBR, mcr and 3'MBR in 10/11 cases. 4/10 cases showed a rearrangement within the MBR that was concordant with the PCR result. A single case, GBS-7, shown to be mcr multiplex positive, gave an inconclusive SB result with the mcr probe.

20 Immunophenotypically this case demonstrated two distinct clonal populations, representing approximately 5% and 15% of the original diagnostic material. The discrepancy between the two techniques in this case probably represents the reduced sensitivity of SB compared with PCR. There was no evidence of a 3'MBR rearrangement in any of the remaining cases by SB.

25 Of the six SB negative FCL cases, a single case; ES-7, showed a t(14;18) using the MBR multiplex. 5/11 FCL cases showed no evidence of a t(14;18) by either SB or PCR. A t(14;18) was detected in two further cases by PCR; FR-6, a case of DLBCL showed an MBR breakpoint and was identified by all 11 laboratories, this finding is compatible with previous studies that have detected a t(14;18) in 20-40% of DLBCL cases.^{161,162} Using the 30 3'MBR multiplex, 10/11 laboratories reported a positive result for sample ES-12, this was a case of Hodgkin's disease which contained very few B cells. It is difficult to explain this result in the absence of an *IGH* rearrangement by Southern blotting. Contamination or incorrect labeling of the sample at source is the most likely explanation.

35 Overall there was excellent concordance throughout the network, although small numbers of both false positive and false negative results were encountered. Overall 12

false positive results were identified, representing less than 0.4% (12/3036) of the total number of analyses. These were reported by five laboratories and involved six of the samples. The majority of the false positives (9/12) were found in three cases. Five false negative results, representing a 6% (5/88) failure rate, were reported by three

5 laboratories, ES-7 was not detected by two laboratories, three further groups within the network commented that this case had shown weak amplification signals with the MBR multiplex. The remaining three false negative cases were reported in isolation by individual laboratories. The results of diagnoses using this approach are very encouraging and suggest that the definition of common approaches and reaction

10 conditions can minimize erroneous results.

Conclusion

In conclusion, we have designed and evaluated a robust three-tube multiplex PCR in order to maximize the detection of the t(14;18). This strategy is capable of amplifying

15 across the breakpoint region in the majority of cases of FCL with a cytogenetically defined translocation. Although the sensitivity of this strategy is lower than conventional single round or nested PCR approaches, it is still perfectly acceptable for diagnostic procedures. The widespread adoption of standardized reagents and methodologies has helped to minimize inaccurate results within this large multi-center

20 network. However, it is noteworthy from the general testing phase of this study that it is impossible to detect a t(14;18) in all cases. This is certainly influenced by additional molecular mechanisms capable of deregulating the BCL2 gene. ^{163, 164}

EXAMPLE 10: Use of DNA extracted from paraffin-embedded tissue biopsies and development of control gene primer set

Background

5 Fresh/frozen tissue is considered to be the ideal sample type for extraction of DNA for use in PCR-based clonality analysis. However, fresh/frozen material is not always available to diagnostic laboratories and in many laboratories throughout Europe, paraffin-embedded tissue samples constitute the majority of diagnostic biopsies submitted for analysis. DNA extracted from paraffin-embedded material is often of poor quality and so PCR protocols need to be evaluated for use with these sample types before 10 they can be widely used in diagnostic laboratories.

The integrity of DNA extracted from paraffin-embedded samples and its amplification by PCR are affected by a number of factors such as thickness of tissue, fixative type, fixative time, length of storage before analysis, DNA extraction procedures and the co-extraction of PCR inhibitors.¹⁶⁵⁻¹⁷² Ten percent neutral buffered formalin (10% 15 NBF) is the most commonly used fixative, although laboratories also use a number of other fixatives, including unbuffered formalin and Bouins. The use of 10% NBF permits the amplification of DNA fragments of a wide range of sizes whereas Bouins appears to be the least amenable for use in PCR analysis.^{167, 168, 171, 173} The integrity of DNA fragments extracted from paraffin-embedded samples also depends on the length of time the blocks 20 have been stored with the best results usually obtained from blocks less than 2 years old, while blocks over 15 years old tend to yield very degraded fragments.¹⁷⁴

Primer design

Initially, five pairs of control gene PCR primers were designed to amplify 25 products of exactly 100, 200, 400, 600 and 1,000 bp in order to assess the quality of DNA submitted for analysis. The target genes were selected on the basis of having large exons with open reading frames to reduce the risk of selecting polymorphic regions and the primers were designed for multiplex usage in the standardized protocols. The following 30 target genes were selected: human thromboxane synthase gene (*TBXAS1*, Exon 9; GenBank Accession No D34621), human recombination activating gene (*RAG1*, Exon 2; GenBank Accession No M29474), human promyelocytic leukemia zinc finger gene (*PLZF*, Exon 1; GenBank Accession No AF060568), and human AF4 gene (Exon 3; GenBank Accession No Z83679, and Exon 11; GenBank Accession No Z83687).

Results of initial testing phase

The primer pairs were tested in separate reactions and subsequently in multiple reactions using high molecular weight DNA. Due to the large size range of the products (100 to 1,000 bp), it was necessary to vary the ratio of primer concentrations to obtain 5 bands of equal intensities in the multiplex reactions. However, it proved extremely difficult to be able to amplify all the bands reproducibly and it was decided that the 1,000 bp product was probably unnecessary, since all the PCR protocols according to the invention give products of less than 600 bp. It was therefore decided to exclude the 1,000 bp product in order to improve the reproducibility of the assay. By increasing the MgCl₂ 10 concentration to 2 mM and adding the primers in a 1:1:1:2 ratio, it was possible to reproducibly amplify four bands (100, 200, 400 and 600 bp) of equal intensity from high molecular weight DNA samples. However, for DNA extracted from paraffin blocks, it was thought that an extra size marker at 300 bp would be extremely informative and that the 600 bp marker might not be necessary. Using the gene sequence for the 1,000 bp 15 marker (*PLZF*), primers were redesigned to generate a 300 bp product. These were tested successfully both in monoplex reactions and in multiplex reactions combining the 100, 200, 300, 400 and 600 bp primers (see Figure 12A).

Thus two primer sets are available for assessing the quality of DNA for amplification: The 100, 200, 300 and 400 bp primers used at 2.5 pmol each can be used 20 for assessing DNA from paraffin-embedded tissues. The addition of the 600 bp primers at 5 pmol allows this set to be used to check the quality of any DNA sample for use with the primers and protocols according to the invention. Both primer sets can be used with ABI Buffer II and 2.0 mM MgCl₂ under standardized amplification conditions. Products can be analyzed on 6% PAGE or 2% agarose (see Figure 12B).

25

Results of general testing phase

Forty five paraffin-embedded biopsies were collected corresponding to 30 of the B-cell malignancies, eight of the T-cell malignancies and seven of the reactive lymphoproliferations submitted as fresh/frozen tissue samples. The age of the paraffin 30 blocks as well as the methods of fixation and embedding of the samples varied between National Networks. The ES samples were submitted as pre-cut sections, NL-14, 15 and 16 were submitted as DNA samples and the remaining biopsies were submitted as paraffin blocks. Five sections (10 µm each) were cut from the paraffin blocks and DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN) following the manufacturer's 35 protocol for isolation of genomic DNA from paraffin-embedded tissue. This method of

DNA extraction was chosen since the kit can be used to rapidly extract DNA from blood, fresh/frozen tissue and paraffin-embedded tissue and thus enables the parallel processing of a variety of sample types with assured quality control. Numerous protocols for extraction of DNA from paraffin-embedded tissue for PCR analysis have been published.^{171, 172, 175-177} Many of these aim to reduce DNA degradation and co-extraction of PCR inhibitors, but many of these methods require prolonged extraction procedures and can be unsuitable for use in the routine diagnostic laboratory.^{166, 178, 179}

DNA sample concentration and integrity were estimated by spectrophotometry and by comparison of sample DNA with known standards on agarose gel electrophoresis. DNA samples (100 ng) were then analyzed for integrity and amplifiability using the control gene PCR primers (100-400 bp) and assessed for clonality at all target loci using the PCR protocols.

In the control gene PCR reaction of 24/45 cases the amplified products were at least 300 bp, whereas in the remaining 21 samples the amplified products were 200 bp or less. No clear correlation between the quality of the DNA and the age of the block or fixation method could be demonstrated. Therefore it is likely that a combination of factors is responsible for the DNA quality in these samples.

The DNA samples were evaluated for clonality using the 18 multiplex PCR reactions and were analyzed by both HD and GS. The number of paraffin samples showing clonality and translocations at the nine target loci were compared with the corresponding fresh/frozen sample data. In samples with control gene PCR products of up to 200 bp, the overall detection of clonality at the nine target loci was 9/55 (16%). Of the 46 missed rearrangements, 45 could be explained by the fact that the expected clonal PCR products had a molecular weight higher than the maximum size amplified by the sample in the control gene PCR. The remaining sample (PT-9) amplified to 100 bp in the control gene PCR but the expected 81 bp TCRG clonal product was not detected. In samples with control gene PCR products of at least 300 bp, the overall detection of clonality at the nine target loci was 42/55 (76%). Of the 13 missed rearrangements, five could again be explained by the fact that the expected clonal PCR products were larger than the maximum size amplified by the sample in the control gene PCR. The remaining eight missed rearrangements could not be explained directly by the quality of the DNA. One false positive clonal result (GBN-9; *IGL*) was detected in a reactive lymph node which may represent pseudoclonality.

PCR inhibitors are known to be present in DNA extracted from paraffin samples. Dilution of the DNA sample may reduce the concentration of these inhibitors to levels

that allow successful amplification to occur. To investigate the effect of diluting DNA samples on the efficiency of amplification, four different concentrations of DNA were tested in the control gene PCR reaction: 5, 50, 100 and 500 ng. We observed that dilution of the DNA samples has a significant effect on the size of the PCR products in the control gene PCR. Overall, 24/45 cases (53%) showed an increased efficiency of amplification when diluted from 100 ng to 50 ng. The optimal DNA concentration appears to be between 50 to 100 ng whereas the use of 500 ng appears to inhibit the amplification of large products (300 bp or above). Although the use of 5 ng of DNA gives acceptable results with the control gene PCR, this can lead to false positivity in PCR-based clonality assays due to the low representation of total lymphoid cell DNA.^{180, 181} More importantly, 5 ng of DNA has no advantage over a dilution to 50 ng of DNA.

To assess whether the use of 50 ng of DNA would also increase the detection of clonality, all the samples were retested at the *IGH* V-J locus using this DNA concentration. The number of clonal rearrangements detected in the three *IGH* V-J tubes using 100 ng of DNA was 12, compared with 23 using the corresponding fresh/frozen samples. The overall detection of clonality at this locus increased to 17 out of 23 when 50 ng of DNA was used, with an additional 9 FR1, 6 FR2 and 4 FR3 clonal products being detected. Thus dilution of the DNA can increase the detection of clonal products, presumably because of dilution of PCR inhibitors. Logically, dilution of DNA is only likely to improve both control gene PCR results and the detection of clonality, if PCR inhibitors are present, not if the DNA sample is highly degraded. Therefore it is recommended that at least two dilutions of DNA are tested using the control gene PCR and that the dilution that gives the better result is used in subsequent clonality analysis.

Nine clonal rearrangements remained undetected after initial analysis, which could not be explained by DNA quality (*TCRG* in PT-9 and NL-11; *TCRB* in GBS-4; *TCRD* in NL-15; *IGK* in GBN-4, NL-4 and NL-5; *IGH* V-J_H in GBS-6 and GBS-8). These samples were retested using 50 ng of DNA, but only one sample (GBS8; *IGH*) showed improved detection, suggesting that other, unknown, factors can prevent amplification of specific targets in a small number of cases. However, it should be noted that for seven of these samples (NL-11, GBS-4, NL-15, GBN-4, NL-5, GBS-6 & GBS-8) clonal products were detected in at least one other locus. This demonstrates that testing for clonality at multiple target loci increases the likelihood of detecting clonal lymphocyte populations.

Conclusion

In conclusion, the protocols as provided herein work well with DNA extracted from paraffin-embedded material provided that the DNA can amplify products of 300 bp or more in the control gene PCR. Two concentrations of DNA are preferably tested in the 5 control gene PCR and the more 'amplifiable' concentration should be used in further testing, although with the proviso that concentrations of DNA less than 20 ng may contribute to the detection of pseudyclonality due to the low representation of target lymphoid DNA.^{180, 181} Overall the data show that assessment of DNA quality using the control gene PCR provides a good indication of the suitability of the DNA for clonality 10 analysis using the protocols provided. It is also important to note that the control gene PCR will give no indication of the amount of lymphoid cell DNA present in the sample and therefore good quality DNA may still produce negative results for clonality analysis. To ensure monoclonal results are reproducible (and to avoid potential pseudyclonality), 15 all clonality assays, particularly using paraffin-extracted DNA, are preferably performed in duplicate and analyzed by HD and GS, wherever possible.

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40

Table 1. B, T, and NK lineage of lymphoid malignancies^a

Lineage	ALL		Chronic lymphocytic leukemias	nodal	Non-Hodgkin lymphomas		Multiple myeloma
	childhood	adult			extra-nodal	skin	
B	82 – 86%	75 – 80%	95 – 97%	95 – 97%	90 – 95%	30 – 40%	100%
T	14 – 18%	20 – 25%	3 – 5%	3 – 5%	5 – 10%	60 – 70%	0%
NK	< 1%	< 1%	1 – 2%	< 2%	< 2%	< 2%	0%

5 a. See Van Dongen et al. 1991¹, Jaffe et al. 2001², and Van Dongen et al. 2002⁵

Table 2. Estimated number of non-polymorphic human V, D, and J gene segments that can potentially be involved in Ig or TCR gene rearrangements^a

Gene segment	<i>IGH</i>	<i>IGK</i>	<i>IGL</i>	<i>TCRA</i>	<i>TCRB</i>	<i>TCRG</i>	<i>TCRD</i>
V segments							
- functional (family)	44 (7) 66 (7) ^b	43 (7) 76 (7)	38 (10) 56 (11)	46 (32) 54 (32)	47 (23) 67 (30)	6 (4) 9 (4)	8 8
- rearrangeable (family)
D segments							
- rearrangeable (family)	27(7)	–	–	–	2	–	3
J segments							
- functional	6 ^c	5 ^d	4	53	13	5	4
- rearrangeable	6 ^c	5 ^d	5 ^e	61	13	5	4

15 a. Only non-polymorphic gene segments with a suitable RSS are included in this table.
b. This estimation does not include the recently discovered (generally truncated) VH pseudogenes, which are clustered in three clans
c. The six JH gene segments are highly homologous over a stretch of ~20 nucleotides, which is sufficient for the design of a consensus primer.

- d. The Jκ segments have a high homology, which allows the design of 2 to 3 Jκ consensus primers.
- e. Five of the seven Jλ gene segments have a suitable RSS.

Table 3. Standardized PCR protocol*Reaction conditions.*

5 • buffer: ABI Buffer II or ABI Gold Buffer
 • 50 µl final volume
 • 100 ng DNA
 • 10 pmol of each primer (unlabeled or 6-FAM labeled)
 (irrespective of total numbers of primers in each multiplex PCR tube)

10 • dNTP: 200 µM final concentration
 • MgCl₂: 1.5 mM final concentration (to be optimized per target)
 • *Taq* enzyme^a: 1U in most tubes; 2U in tubes with many primers (>15)

15

Cycling conditions

• pre-activation 7 min. at 95°C
 • annealing temperature: 60°C
 • cycling times:

		"classical" <i>PCR equipment</i>	"newer" <i>PCR equipment</i>
	-denaturation	45 sec.	30 sec.
	-annealing	≥45 sec.	≥30 sec.
	-extension	1.30 min.	≥30 sec.
	-final extension	≥10 min.	≥10 min.

25 • number of cycles: 35
 • hold 15°C (or room temperature)

30 a. AmpliTaq Gold (Applied Biosystems, Foster City, CA) was used in combination with
 1x ABI Buffer II or 1x ABI Gold Buffer (Applied Biosystems), depending on the target.

35

Table 4. Standardized protocol for heteroduplex analysis of PCR products

PCR product preparation

5

- tube with 10-20 µl of PCR product
- denaturation of PCR product: 5 min. at 95°C
- re-annealing of PCR product: 60 min. at 4°C

Electrophoresis conditions (non-commercial polyacrylamide gels)

10

- gel: 6% non-denaturing polyacrylamide (acrylamide: bisacrylamide 29:1)
- buffer: 0.5 x TBE
- loading buffer: 5 µl ice-cold non-denaturing bromophenol blue loading buffer
- electrophoresis: typically 2-3 hours at 110 V or overnight at 40-50 V^a

15 *Electrophoresis conditions (commercial polyacrylamide gels)*

- gel: non-denaturing polyacrylamide (e.g. BioRad PreCast Gel System or Amersham Pharmacia Biotech Gene Gel Excel Kit)
- buffer: 1 x TBE
- loading buffer: ice-cold non-denaturing bromophenol blue loading buffer

20 • electrophoresis: 1,5 hours at 100 V

Visualization

- staining: 5-10 min. in 0.5 µg/ml EtBr in H₂O
- destaining / washing: 2x 5-10 min. in H₂O

25 • visualization: UV illumination

- alternative: silver staining using Amersham Pharmacia Biotech DNA Silver stain kit

a. Voltage and electrophoresis time depend on PCR amplicon sizes, thickness of

30 polyacrylamide gel, and type of PCR equipment, and should be adapted accordingly.

Table 5. Standardized protocol for GeneScanning of PCR products**A. Gel-based sequencers**

5

PCR product preparation

1. PCR product dilution: initially 1:10 in formamide or H₂O (can be altered if fluorescent signal is outside optimal range; see electrophoresis conditions)
2. sample volume: 2 µl diluted PCR product
3. loading buffer volume: 0.5 µl blue dextran loading buffer + 0.5 µl TAMRA internal standard + 2 µl deionized formamide
4. denaturation of PCR product: 2 min. at 95°C or higher temperature
5. cooling of PCR product at 4°C

10

Electrophoresis conditions

6. gel: 5% denaturing polyacrylamide
7. buffer: 1 x TBE
8. electrophoresis: 2-3.5 hours^a (see Table 25)
9. optimal fluorescent signal intensity:
 - 600-4,000 fluorescent units (373 platforms)
 - 400-7,000 fluorescent units (377 platforms)

15

B. Capillary sequencers (to be optimized per sequencer)

20

PCR product preparation

1. 1 µl PCR product (volume of PCR product or sampling times can be altered if fluorescent signal is outside optimal range; see electrophoresis conditions)
2. sample volume: 1 µl PCR product + 9.5 µl (Hi-Di) formamide + 0.5 µl ROX-400 heteroduplex analysis internal standard
3. denaturation of PCR product: 2 min. at 95°C or higher temperature
4. cooling of PCR product at 4°C for an hour

25

Electrophoresis conditions

5. gel: 3100 POP4 polymer
6. buffer: 1x 3100 buffer with EDTA
7. electrophoresis: 45 minutes^b
8. optimal fluorescent signal intensity:
 - up to 10,000 fluorescent units

30

^a Electrophoresis time depends on amplicon sizes and on employed platform.

^b For 36 cm capillary; time taken depends on capillary used.

35

Table 6. Sensitivity of detection of clonal TCRB rearrangements

TCRB tube	Involved primer pair		Clonal Control	Size of PCR product	Sensitivity of detection	
	V	J			single PCR ^a	multiplex PCR
tube A	V β 2	J β 1.2	patient	261 nt	1-5%	5%
	V β 2	J β 1.3	patient	267 nt	5%	5%
	V β 2	J β 1.6	patient	267 nt		<5%
	V β 7	J β 2.2	patient	254 nt		10%
	V β 8a	J β 1.2	Jurkat	267 nt	0.1%	0.5 - 1 %
	V β 8a	J β 2.7	patient	264 nt		10%
	V β 10	J β 2.7	PEER	263 nt		20%
	V β 3/12a/13a/1					
	5	J β 1.6	patient	278 nt	<5%	5%
	V β 3/12a/13a/1					
	5	J β 2.7	patient	286 nt		10%
	RPMI-					
	V β 17	J β 2.7	8402	260 nt		10%
	V β 17	J β 1.1	patient	260 nt	1%	10%
	V β 18	J β 1.2	DND41	261 nt	1%	10%
	V β 22	J β 1.1	patient	265 nt	0.1%	10%
	V β 8b/23	J β 1.2	H9	257 nt	0.1%	0.5%
	RPMI-					
	V β 24	J β 1.5	8402	264 nt	0.5%	10%
tube B	V β 2	J β 2.1	Molt-4	267 nt	5%	5%
	V β 1/5	J β 2.1	patient	266 nt	5%	1-5%
	V β 6a/11	J β 2.1	patient	265 nt	1%	5%
	V β 6a/11	J β 2.5	patient	258 nt		5%
	V β 7	J β 2.3	PEER	271 nt		<5%
	V β 8a	J β 2.1	patient	293 nt	0.1%	1%
	V β 3/12a/13a/1					
	5	J β 2.1	patient	258 nt	5%	10%
	V β 3/12a/13a/1					
	5	J β 2.3	patient	258 nt		<5%
	V β 16	J β 2.1	patient	258 nt	0.5%	10%
	V β 17	J β 2.5	CML-T1	270 nt	0.1 - 1%	1%
	V β 21	J β 2.3	patient	282 nt	0.5%	<10%
tube C	D β 1	J β 1.1	patient	304 nt	0.10%	<5%
	D β 1	J β 1.2	patient	306 nt	5%	5%
	D β 1	J β 1.4	patient	310 nt		5-10%
	D β 1	J β 1.6	patient	320 nt		20%
	D β 1	J β 2.1	patient	309 nt	5%	20%
	D β 1	J β 2.7	patient	307 nt		<5%
	D β 1	J β 2.5	patient	310 nt		<1%
	D β 2	J β 1.4	patient	182 nt		<1%
	D β 2	J β 2.1	patient	185 nt	1%	<5%

TCRB tube	Involved primer pair		Clonal Control	Size of PCR product	Sensitivity of detection	
	V	J			single PCR ^a	multiplex PCR
Dβ2	Jβ2.5	patient		191 nt		5%

a. The dilution experiment for assessing the sensitivity of the single PCR was not performed in each case.

Table 7. Sensitivity of detection of clonal TCRD gene rearrangements

TCRD rearrangement	Clonal control sample (approximate size)	Sensitivity of detection by heteroduplex
Vδ1-Jδ1	patient (200 nt)	5%
	patient (190 nt)	1-5%
	patient (200 nt)	5%
Vδ2-Jδ1	patient (200 nt)	5%
	patient (220 nt)	5%
	patient (210 nt)	5%
Vδ2-Jδ3	patient (220 nt)	5%
Vδ3-Jδ1	patient (270 nt)	5%
Vδ6-Jδ2	Loucy (210 nt)	0.5%
	patient (210 nt)	10 %
	Loucy (150 nt)	0.2%
Dδ2-Jδ1	patient (160 nt)	0.5%
	patient (135 nt)	0.5%
	patient (150 nt)	5%
Dδ2-Jδ3	NALM-16(170 nt)	1%
Dδ2-Dδ3	patient (200 nt)	1%
	patient (190 nt)	0.5%
	patient (170 nt)	0.5%
Vδ2-Dδ3	REH (240 nt)	5-10%
	NALM-16 (230 nt)	1-5%
	patient (250 nt)	5%

5

Table 8. Concordance between multiplex PCR results and Southern blot (SB) analysis results (PCR/SB) on Ig/TCR gene rearrangements per (sub)category of included frozen samples

10

Diagnosis	IGH^a	IGK	IGL	TCRB	TCRG	TCRD
pre-follicular (n=8)	C ^b : 8/8 P ^b : 0/0	C: 8/8 P: 0/0	C: 4/4 P: 4/4	C: 2/4 ^b P: 4/4	C: 0/0 P: 8/8	C: 0/0 P: 8/8 ^e
B-CLL (n=16)	C: 15/16 P: 0/0	C: 16/16 P: 0/0	C: 5/5 P: 9/11	C: 1/1 P: 15/15	C: 0/0 P: 16/16	C: 2/2 P: 14/14
(post-)follicular (n=25)	C: 22/25 ^b P: 0/0	C: 19/24 ^c P: 0/1	C: 3/5 P: 19/20	C: 2/4 P: 21/21 ^{d,e}	C: 0/1 P: 22/24	C: 0/0 P: 24/25 ^e
All B-cell malignancies (n=49)	C: 45/49 P: 0/0	C: 43/48 P: 0/1	C: 12/14 P: 32/35	C: 4/8 P: 41/41	C: 0/1 P: 46/48	C: 2/2 P: 46/47
T-cell malignancies (n=18)	C: 2/2 P: 15/16 ^e	C: 0/0 P: 17/18	C: 0/0 P: 17/18	C: 17/17 ^c P: 1/1	C: 15/16 ^b P: 1/2	C: 2/3 P: 14/15 ^e
Reactive samples (n=15)	C: 0/0 P:	C: 0/0 P:	C: 0/0 P:	C: 0/0 P: 14/15	C: 0/0 P:	C: 0/0 P:

	15/15	15/15	15/15		15/15	15/15
Miscellaneous (n=8)	C: 3/3 P: 3/5	C: 2/2 P: 4/6	C: 0/0 P: 6/8	C: 3/3 P: 5/5 ^{a,d}	C: 1/1 P: 6/7	C: 1/1 P: 5/7
All samples (n=90)	C: 50/54 P: 33/36	C: 45/50 P: 36/40	C: 12/14 P: 70/76	C: 25/29 P: 60/61	C: 16/18 P: 68/72	C: 5/6 P: 80/84

a. Includes both VH-JH and DH-JH PCR analysis
b. C, clonal rearrangements; P, polyclonal rearrangements
c. In one sample clonality in GeneScanning only
5 d. In one sample clonality in heteroduplex analysis only
e. In one sample polyclonality in GeneScanning only
f. In one sample polyclonality in heteroduplex analysis only

Table 9. Complementarity of different Ig multiplex PCR targets for clonality detection in Southern blot-defined B-cell malignancies

Multiplex PCR tubes	Diagnosis ^a			
	Pre-germinal center B (n=8)	B-CLL (n=16)	(post-)germinal center B (n=25)	all B-cell malignancies (n=49)
<i>IGH</i> VH-JH FR1	8/8 ^b (100 %)	14/16 ^c (88 %)	15/25 ^b (60 %)	37/49 (76 %)
<i>IGH</i> VH-JH FR2	8/8 (100 %)	15/16 (94 %)	14/25 ^b (56 %)	37/49 (76 %)
<i>IGH</i> VH-JH FR3	8/8 (100 %)	14/16 (88 %)	11/25 ^c (44 %)	33/49 (67 %)
<i>IGH</i> VH-JH 3FR	8/8 (100 %)	15/16 (94 %)	17/25 (68 %)	40/49 (82 %)
<i>IGH</i> DH-JH	0/8 (0 %)	11/16 (69 %)	11/25 (44 %)	22/49 (45 %)
<i>IGH</i> VH-JH + <i>IGH</i> DH-JH	8/8 (100 %)	15/16 (94 %)	22/25 (88 %)	45/49 (92 %)
<i>IGK</i>	8/8 (100 %)	16/16 (100 %)	21/25 ^d (84 %)	45/49 (92 %)
<i>IGL</i>	4/8 (50 %)	7/16 ^e (44 %)	4/25 ^f (16 %)	15/49 (31 %)
<i>IGH</i> VH-JH + <i>IGK</i>	8/8 (100 %)	16/16 (100 %)	21/25 (84 %)	45/49 (92 %)
<i>IGH</i> VH-JH + <i>IGL</i>	8/8 (100 %)	15/16 (94 %)	17/25 (68 %)	40/49 (82 %)
<i>IGH</i> VH-JH + <i>IGH</i> DH-JH + <i>IGK</i>	8/8 (100 %)	16/16 (100 %)	24/25 (96 %)	48/49 (98 %)
<i>IGH</i> VH-JH + <i>IGH</i> DH-JH + <i>IGK</i> + <i>IGL</i>	8/8 (100 %)	16/16 (100 %)	24/25 (96 %)	48/49 (98 %)

5 a. All samples have clonal gene rearrangements in at least the *IGH* locus as determined by Southern blot analysis

b. Two cases showed clonal products in GeneScanning, but polyclonal products in heteroduplex analysis

c. One case showed clonal products in GeneScanning, but polyclonal products in heteroduplex analysis

10 d. Including case 25-NL-4 with weak clonal *IGH* but polyclonal *IGK* gene rearrangements in Southern blot analysis

e. Including cases 11-NL-19 and 12-ES-1 with clonal *IGH* + *IGK* but polyclonal *IGL* gene rearrangements in Southern blot analysis

15

20

Table 10. Conditions and control samples for multiplex PCR analysis of Ig / TCR gene rearrangements and translocations t(11;14) and t(14;18)

Multiplex PCR	Tubes	PCR conditions			Positive controls (examples)	
		Buffer	TaqGold (U)	MgCl ₂ (mM)	polyclonal	monoclonal ^a
<i>IGH</i> VH-JH	A / B / C	Gold / II	1	1.5	tonsil	A: NALM-6; SU-DHL-5; SU-DHL-6 B: NALM-6; SU-DHL-5; SU-DHL-6 C: NALM-6; SU-DHL-5; SU-DHL-6
<i>IGH</i> DH-JH	D / E	Gold	1	1.5	tonsil	D: KCA; ROS15 E: HSB-2, HPB-ALL
<i>IGK</i>	A / B	Gold / II	1	1.5	tonsil	A: KCA; ROS15 B: ROS15, 380
<i>IGL</i>	A	Gold / II	1	2.5	tonsil	A: CLL-1; EB-4B; KCA
<i>TCRB</i>	A / B / C	II	2 (A,B) ^b 1 (C)	3.0 (A,B) 1.5 (C)	PB-MNC ^c	A: RPMI-8402; Jurkat; PEER; DND-41 B: PEER; CML-T1, MOLT-3 C: Jurkat
<i>TCRG</i>	A / B	II	1	1.5	PB-MNC ^c	A: MOLT-3; RPMI-8402; Jurkat; PEER B: Jurkat; PEER
<i>TCRD</i>	A	II	1	2.0	PB-MNC ^c	A: PEER, REH
<i>BCL1-IGH</i>	A	II	1	2.0	NA ^c	A: JVM 2
<i>BCL2-IGH</i>	A / B / C	II	1	1.5	NA ^c	A: DoHH2; SU-DHL-6 B: K231 ^d

a. Most clonal cell line controls can be obtained via the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; contact person: dr. H.G. Drexler (address: Department of Human and Animal Cell Cultures, Mascheroder Weg 1B, 38124 Braunschweig, Germany). ^{192, 193}

5 b. In most multiplex tubes only 1 U *Taq*Gold is needed, but 2 U *Taq*Gold are needed in *TCRB* tubes A and B because they contain >15 different primers.

c. Abbreviations: PB-MNC, mononuclear cells from peripheral blood; NA, not applicable.

d. The t(14;18) positive cell lines K231, OZ, and SC1 were kindly provided by prof.

10 Martin Dyer, University of Leicester, Leicester, GB.

Table 11. Size ranges, non-specific bands, and detection method in multiplex PCR analysis of Ig/TCR gene rearrangements and chromosome aberrations t(11;14) and t(14;18)

Multiplex PCR	Size range (bp)	Nonspecific bands (bp)	Preferred method of analysis	GeneScan running time: gel/capillary
<i>IGH</i> VH-JH	Tube A: 310-360 Tube B: 250-295 Tube C: 100-170	Tube A: ~85 Tube B: - Tube C: -	GeneScanning and heteroduplex analysis equally suitable	3 – 3.5 h / 45 min
<i>IGH</i> DH-JH	Tube D: 110-290 (D _H 1/2/4/5/6-J _H) +390-420 (D _H 3-J _H) Tube E: 100-130	Tube D: 350 ^a Tube E: 211 ^b	heteroduplex analysis slightly preferred over GeneScanning (variation of product sizes hampers GeneScanning)	3 – 3.5 h / 45 min
<i>IGK</i>	Tube A: 120-160 (V _K 1f/6/V _K 7-J _K) +190-210 (V _K 3f-J _K) +260-300 (V _K 2f/V _K 4/V _K 5-J _K) Tube B: 210-250 V _K 1f/6/V _K 7-Kde +270-300 (V _K 3f/intron-Kde) +350-390 (V _K 2f/V _K 4/V _K 5-Kde)	Tube A: - Tube B: ~404	heteroduplex analysis slightly preferred over GeneScanning (small junction size + variation of product sizes hampers GeneScanning)	3 – 3.5 h / 45 min
<i>IGL</i>	Tube A: 140-165	Tube A: -	heteroduplex analysis clearly preferred over GeneScanning (small junction size hampers GeneScanning)	2 h / 45 min
<i>TCRB</i>	Tube A: 240-285 Tube B: 240-285 Tube C: 170-210 (D _B 2) + 285-325 (D _B 1)	Tube A: (273) ^c Tube B: <150, 221 ^d Tube C: 128, 337 ^d	heteroduplex analysis slightly preferred over GeneScanning (limited repertoire, particularly in case of ψ V γ 10 and ψ V γ 11 usage)	2 h / 45 min

<i>TCRG</i>	Tube A: 145-255 Tube B: 80-220	Tube A: - Tube B: -	GeneScanning and heteroduplex analysis equally suitable	2 h / 45 min
<i>TCRD</i>	Tube A: 120-280	Tube A: ~90	heteroduplex analysis clearly preferred over GeneScanning (low amount of template + variation of product sizes hampers GeneScanning)	2 h / 45 min
<i>BCL1- IGH</i>	Tube A: 150-2000	Tube A: ~550 (weak)	agarose	NA ^e
<i>BCL2- IGH</i>	Tube A: variable Tube B: variable Tube C: variable	Tube A: - Tube B: - Tube C: -	agarose	NA ^e

a. The nonspecific 350 bp band is the result of cross-annealing of the D_{H2} primer to a sequence in the region upstream of J_{H4} . In GeneScanning this nonspecific band does not comigrate with D-J products (see Figure 5B).

5 b. The 211 bp PCR product represents the smallest background band derived from the germline D_{H7} - J_{H1} region. When the PCR amplification is very efficient, also longer PCR products might be obtained because of primer annealing to downstream J_H gene rearrangements; e.g. 419 bp (D_{H7} - J_{H2}), 1031 bp (D_{H7} - J_{H3}), etc.

10 c. The 273 bp band (mainly visible by GeneScanning) is particularly seen in samples with low numbers of contaminating lymphoid cells.

d. Intensity of non-specific band depends on primer quality.

e. NA, not applicable

CLAIMS

1. A set of nucleic amplification primers capable of amplifying a V_H - J_H *IGH* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the V_H family primers shown in Fig. 3B, or a variant thereof, and wherein said reverse primer is the J_H consensus primer shown in Fig. 3B, or a variant thereof.
5
2. A set of nucleic amplification primers capable of amplifying a D_H - J_H *IGH* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the D_H family primers shown in Fig. 4A, or a variant thereof, and wherein said reverse primer is the J_H consensus primer shown in Fig. 4A, or a variant thereof.
10
3. A set of nucleic amplification primers capable of amplifying a V_K - J_K *IGK* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the V_K family primers shown in Fig. 5B, or a variant thereof, and wherein said reverse primer is a J_K primer shown in Fig. 5B, or a variant thereof.
15
4. A set of nucleic amplification primers capable of amplifying a V_K -intron- Kde *IGK* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the V_K primers or the INTR primer shown in Fig. 5B, or a variant thereof, and wherein said reverse primer is the Kde primer shown in Fig. 5B, or a variant thereof.
20
5. A set of nucleic amplification primers capable of amplifying a V_λ - J_λ *IGL* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the V_λ primers shown in Fig. 6B, or a variant thereof, and wherein said reverse primer is the J_λ primer shown in Fig. 6B, or a variant thereof.
25
6. A set of nucleic amplification primers capable of amplifying a V_β - J_β *TCRB* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the V_β family primers shown in Fig. 7B, or a variant thereof, and wherein said reverse primer is selected from the $J_\beta A$ en $J_\beta B$ primers shown in Fig. 7B, or a variant thereof.
30
7. A set of nucleic amplification primers capable of amplifying a D_β - J_β *TCRB* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the D_β primers shown in Fig. 7B, or a variant thereof, and wherein said reverse primer is selected from the $J_\beta A$ en $J_\beta B$ primers shown in Fig. 7B, or a variant thereof.
35

8. A set of nucleic amplification primers capable of amplifying a *V γ -J γ* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the *V γ* family primers shown in Fig. 8B, or a variant thereof, and wherein said reverse primer is selected from the *J γ* primers shown in Fig. 8B, or a variant thereof.

9. A set of nucleic amplification primers capable of amplifying a *V δ -J δ TCRD* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the *V δ* primers shown in Fig. 9B, or a variant thereof, and wherein said reverse primer is selected from the *J δ* primers shown in Fig. 9B, or a variant thereof.

10. A set of nucleic amplification primers capable of amplifying a *D δ -D δ TCRD* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is the *D δ 2* primer shown in Fig. 9B, or a variant thereof, and wherein said reverse primer is the *D δ 3* primer shown in Fig. 9B, or a variant thereof.

15. 11. A set of nucleic amplification primers capable of amplifying a *D δ -J δ TCRD* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is the *D δ 2* primer shown in Fig. 9B, or a variant thereof, and wherein said reverse primer is selected from the *J δ* primers shown in Fig. 9B, or a variant thereof.

12. 12. A set of nucleic amplification primers capable of amplifying a *V δ -D δ TCRD* rearrangement comprising a forward primer and a reverse primer, wherein said forward primer is selected from the *V δ* primers shown in Fig. 9B, or a variant thereof, and wherein said reverse primer is the *D δ 3* primer shown in Fig. 9B, or a variant thereof.

20. 13. A set of nucleic amplification primers capable of amplifying a chromosomal translocation (11;14)(*BCL1-IGH*) comprising a forward primer and a reverse primer, wherein said forward primer is the *BCL1/MTC* primer as shown in Fig. 10A, or a variant thereof, and wherein said reverse primer is the *JH* consensus primer shown in Fig. 10A, or a variant thereof.

25. 14. A set of nucleic amplification primers capable of amplifying a chromosomal translocation t(14;18)(*BCL2-IGH*), comprising a forward primer and a reverse primer, wherein said forward primer is selected from the *MBR* primers, the *3'MBR* primers and the *mcr* primers shown in Fig. 11A, or a variant thereof, and wherein said reverse primer is the *JH* consensus primer shown in Fig. 11A, or a variant thereof.

30. 15. A set of nucleic amplification primers capable of amplifying the human *TBXAS1* gene comprising a forward and a reverse primer, wherein said forward primer is the

TBXAS1/X9U primer of Fig. 12 A, or a variant thereof, and wherein said reverse primer is the TBXAS1/X9L primer of Fig. 12A, or a variant thereof.

16. A set of nucleic amplification primers capable of amplifying the human recombination activating protein (*RAG1*) gene comprising a forward and a reverse primer, wherein said forward primer is the RAG1/X2U primer of Fig. 12A, or a variant thereof, and wherein said reverse primer is the RAG1/X2L primer of Fig. 12A, or a variant thereof.

17. A set of nucleic amplification primers capable of amplifying human promyelocytic leukemia zinc finger protein (*PLZF*) comprising a forward and a reverse primer, wherein said forward primer is the PLZF/X1U primer of Fig. 12A, or a variant thereof, and wherein said reverse primer is the PLZF/X1L primer of Fig. 12A, or a variant thereof.

18. A set of nucleic amplification primers capable of amplifying gene the human *AF4* gene (Exon 3) comprising a forward and a reverse primer, wherein said forward primer is the AF4/X3U primer of Fig. 12A, or a variant thereof, and wherein said reverse primer is the AF4/X3L primer of Fig. 12A, or a variant thereof.

19. A set of nucleic amplification primers capable of amplifying gene the human *AF4* gene (Exon 11) comprising a forward and a reverse primer, wherein said forward primer is the AF4/X11U primer of Fig. 12A, or a variant thereof, and wherein said reverse primer is the AF4/X11L primer of Fig. 12A, or a variant thereof.

20. A nucleic acid amplification assay, preferably a PCR assay, more preferably a multiplex PCR assay, using at least one set of primers according to any one of claims 1 to 19.

21. A method for detecting a $V_{H\text{-}}J_{H\text{-}}$ *IGH* rearrangement, comprising using one or more sets of primers according to claim 1 in a nucleic acid amplification assay according to claim 20.

22. A method for detecting a $D_{H\text{-}}J_{H\text{-}}$ *IGH* rearrangement, comprising using one or more sets of primers according to claim 2 in a nucleic acid amplification assay according to claim 20.

23. A method for detecting a $V_{K\text{-}}J_{K\text{-}}$ *IGK* rearrangement, comprising using one or more sets of primers according to claim 3 in a nucleic acid amplification assay according to claim 20.

24. A method for detecting a $V_{K\text{-}}\text{intron-}K_{de}$ *IGK* rearrangement, comprising using one or more sets of primers according to claim 4 in a nucleic acid amplification assay according to claim 20.

25. A method for detecting a $\text{V}\Lambda\text{-J}\Lambda$ TGL rearrangement, comprising using one or more sets of primers according to claim 5 in a nucleic acid amplification assay according to claim 20.

26. A method for detecting a $\text{V}\beta\text{-J}\beta$ TCRB rearrangement, comprising using one or 5 more sets of primers according to claim 6 in a nucleic acid amplification assay according to claim 20.

27. A method for detecting a $\text{D}\delta\text{-J}\delta$ TCRB rearrangement, comprising using one or more sets of primers according to claim 7 in a nucleic acid amplification assay according to claim 20.

10 28. A method for detecting a $\text{V}\gamma\text{-J}\gamma$ TCRG rearrangement, comprising using one or more sets of primers according to claim 8 in a nucleic acid amplification assay according to claim 20.

29. A method for detecting a $\text{V}\delta\text{-J}\delta$ TCRD rearrangement, comprising using one or more sets of primers according to claim 9 in a nucleic acid amplification assay according 15 to claim 20.

30. A method for detecting a $\text{D}\delta\text{-D}\delta$ TCRD rearrangement, comprising using one or more sets of primers according to claim 10 in a nucleic acid amplification assay according to claim 20.

20 31. A method for detecting a $\text{D}\delta\text{-J}\delta$ TCRD rearrangement, comprising using one or more sets of primers according to claim 11 in a nucleic acid amplification assay according to claim 20.

32. A method for detecting a $\text{V}\delta\text{-D}\delta$ TCRD rearrangement, comprising using one or more sets of primers according to claim 12 in a nucleic acid amplification assay according to claim 20.

25 33. A method for detecting a chromosomal translocation (11;14)(*BCL1-IGH*); comprising using one or more sets of primers according to claim 13 in a nucleic acid amplification assay according to claim 20.

34. A method for detecting a chromosomal translocation t(14;18)(*BCL2-IGH*), comprising using one or more sets of primers according to claim 14 in a nucleic acid 30 amplification assay according to claim 20.

35. A method for detecting a gene selected from the group consisting of the human *AF4* gene (exon 3), the human *AF4* gene (exon 11), the human *PLZF1* gene, the human *RAG1* gene and the human *TBXAS1* gene, comprising using one or more sets of primers according to any one of claims 15 to 19 in a nucleic acid amplification assay according to claim 20.

36. Use of a method according to claim 35 to assess the quality of a DNA sample extracted from a biological sample, preferably a paraffin-embedded biological sample.

37. A method for detecting two or more rearrangements, two or more translocations or at least one rearrangement and at least one translocation selected from the group

5 consisting of a $V_{H-J_H} IGH$ rearrangement, a $D_{H-J_H} IGH$ rearrangement, a $V_{K-J_K} IGK$ rearrangement, a $V_{K/intron-Kde} IGK$ rearrangement, a $V_{\Lambda-J_{\Lambda}} IGL$ rearrangement, a $V_{\delta-J_{\delta}} TCRB$ rearrangement, a $D_{\delta-J_{\delta}} TCRB$ rearrangement, a $V_{\gamma-J_{\gamma}} TCRG$ rearrangement, a $V_{\delta-J_{\delta}} TCRD$ rearrangement, a $D_{\delta-D_{\delta}} TCRD$ rearrangement, a $D_{\delta-J_{\delta}} TCRD$ rearrangement, a $V_{\delta-D_{\delta}} TCRD$ rearrangement, a $t(11;14)(BCL1-IGH)$ translocation and
10 $t(14;18)(BCL2-IGH)$ translocation, using at least two sets of primers according to any one of claims 1 or 14.

38. A method for assessing clonal rearrangements and/or chromosome aberrations using at least one set of primers according to any one of claims 1 to 14, and optionally at least one set of primers according to any one of claims 15 to 19.

15 39. A method according to claim 38 for the detection of minimal residual disease (MRD) or for identification of PCR targets to be used for MRD detection via real-time quantitative PCR.

40. A method according to claim 38 or 39, wherein an amplified nucleic acid is detected using automated high resolution PCR fragment analysis.

20 41. A kit for the detection of at least one rearrangement selected from the group consisting of a $V_{H-J_H} IGH$ rearrangement, a $D_{H-J_H} IGH$ rearrangement, a $V_{K-J_K} IGK$ rearrangement, a $V_{K/intron-Kde} IGK$ rearrangement, a $V_{\Lambda-J_{\Lambda}} IGL$ rearrangement, a $V_{\delta-J_{\delta}} TCRB$ rearrangement, a $D_{\delta-J_{\delta}} TCRB$ rearrangement, a $V_{\gamma-J_{\gamma}} TCRG$ rearrangement, a $V_{\delta-J_{\delta}} TCRD$ rearrangement, a $D_{\delta-D_{\delta}} TCRD$ rearrangement, a $D_{\delta-J_{\delta}} TCRD$ rearrangement, a $V_{\delta-D_{\delta}} TCRD$, and/or at least one translocation selected from
25 $t(11;14)(BCL1-IGH)$ and $t(14;18)(BCL2-IGH)$, comprising at least one set of primers according to any one of claims 1 to 14.

42. A kit according to claim 41, further comprising at least one set of primers according to any one of claims 15 to 19.

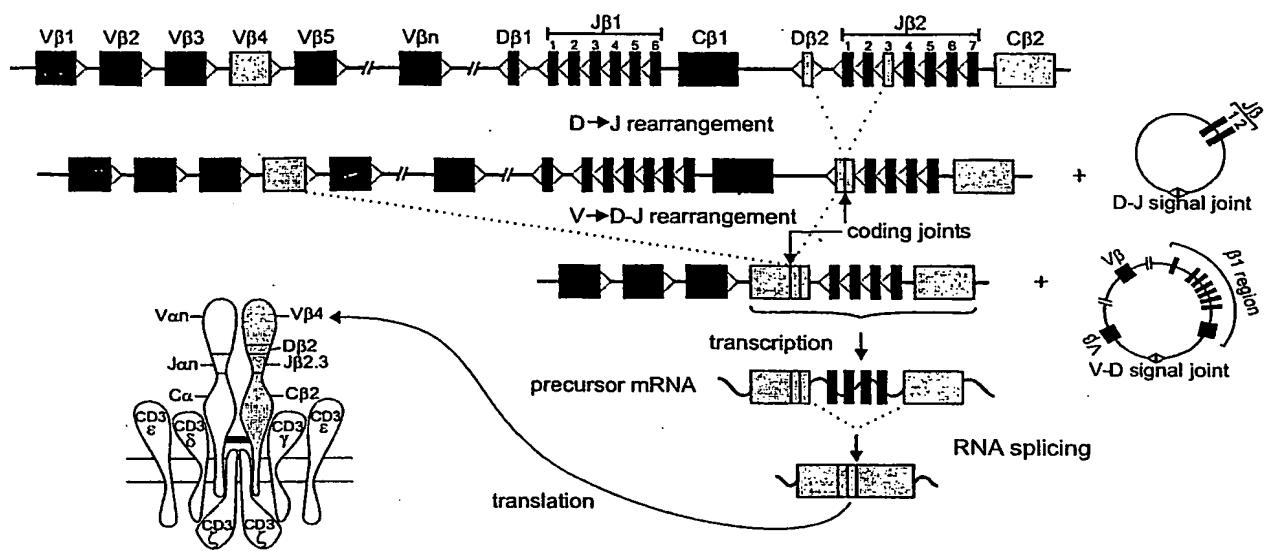


Figure 1

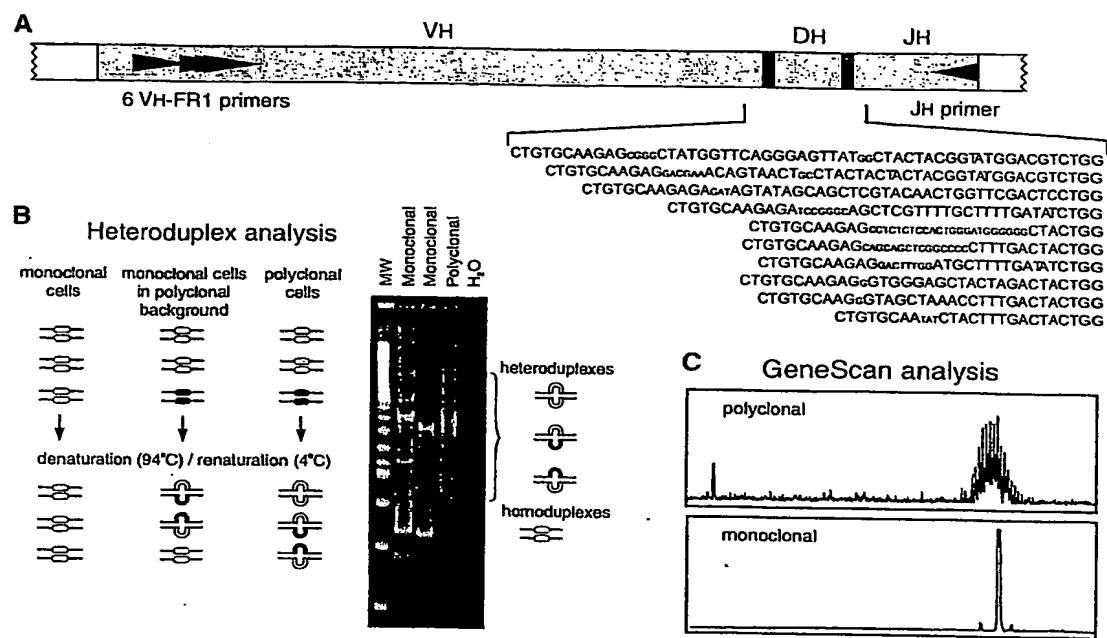
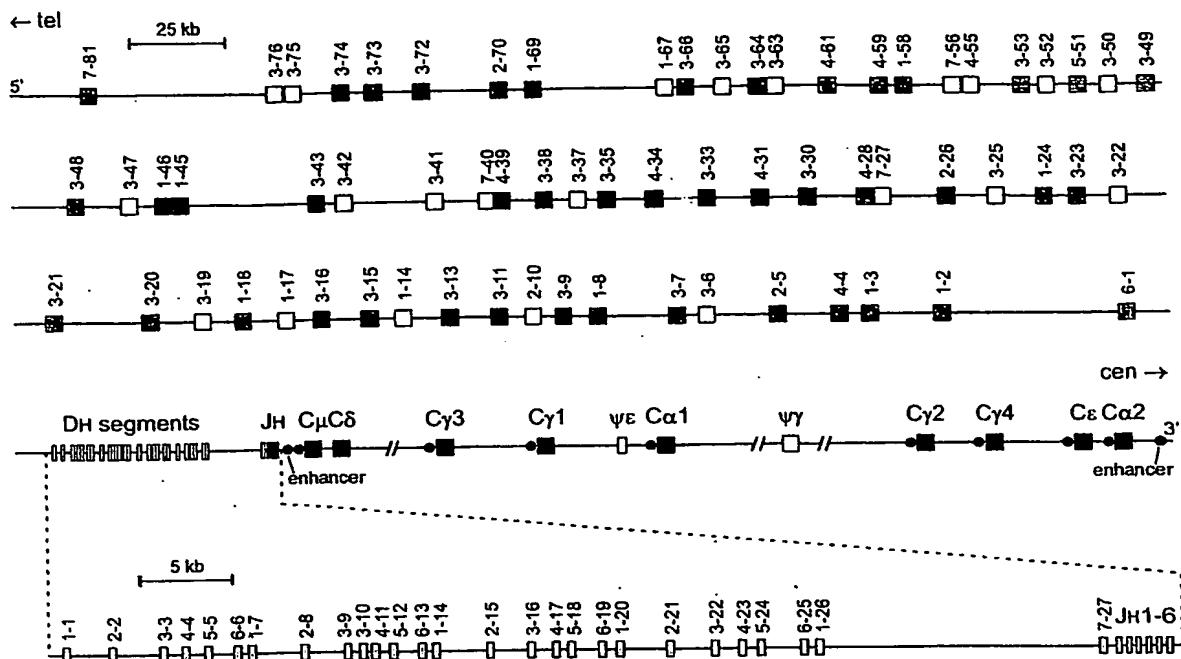


Figure 2

A. *IGH* gene complex (#14q32.3)



B

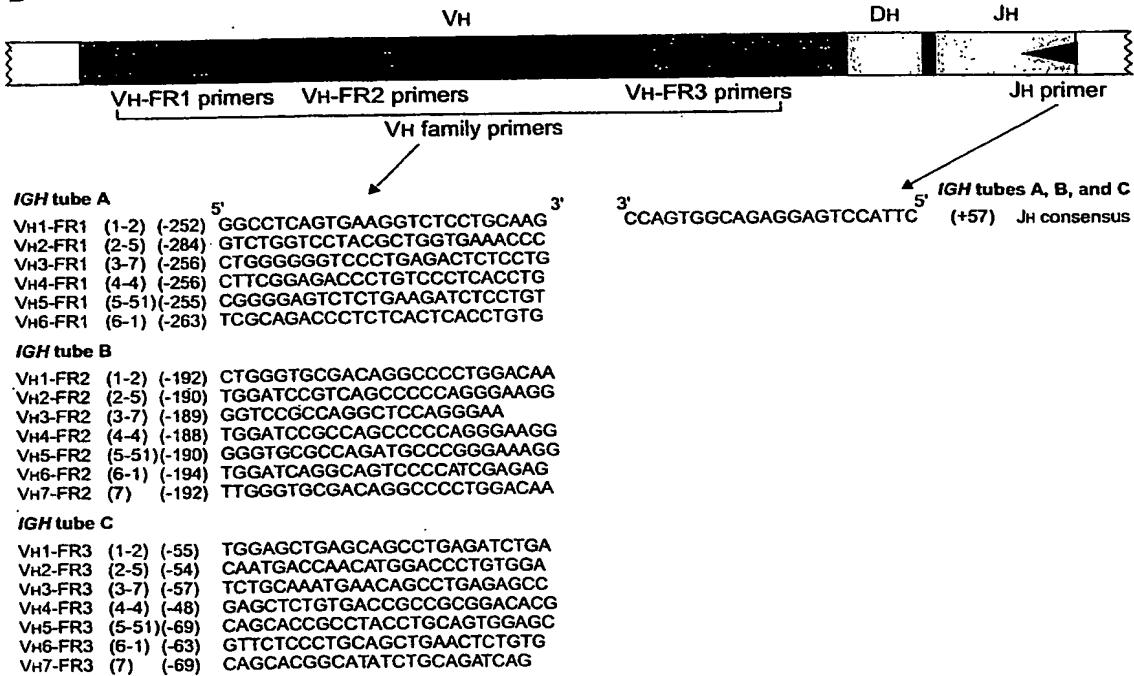


Figure 3 (A and B)

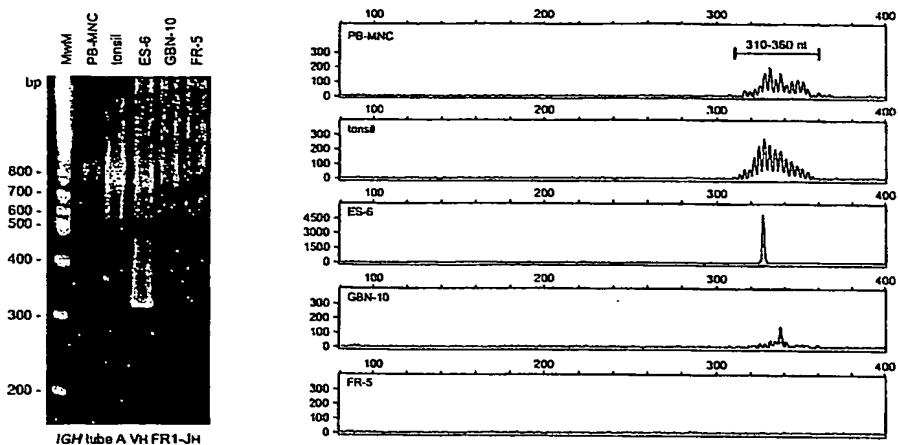
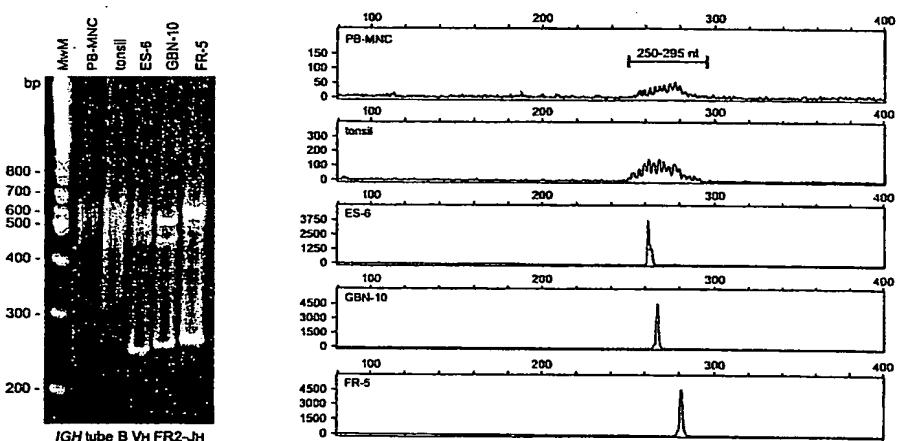
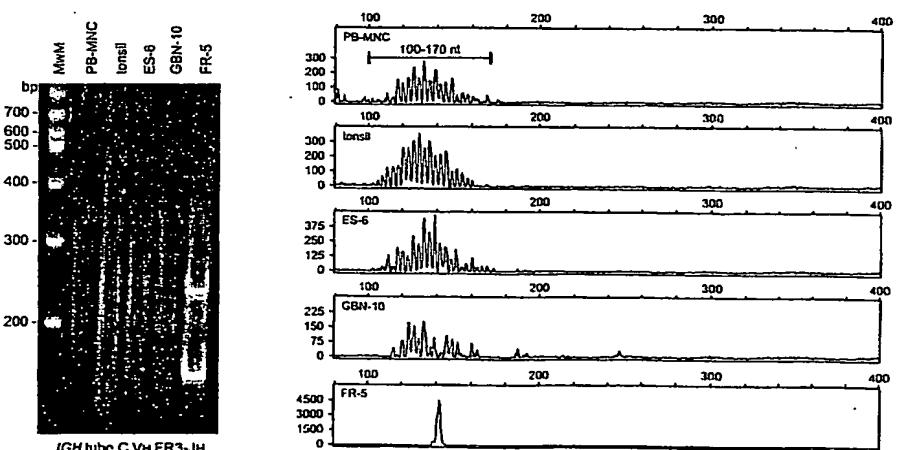
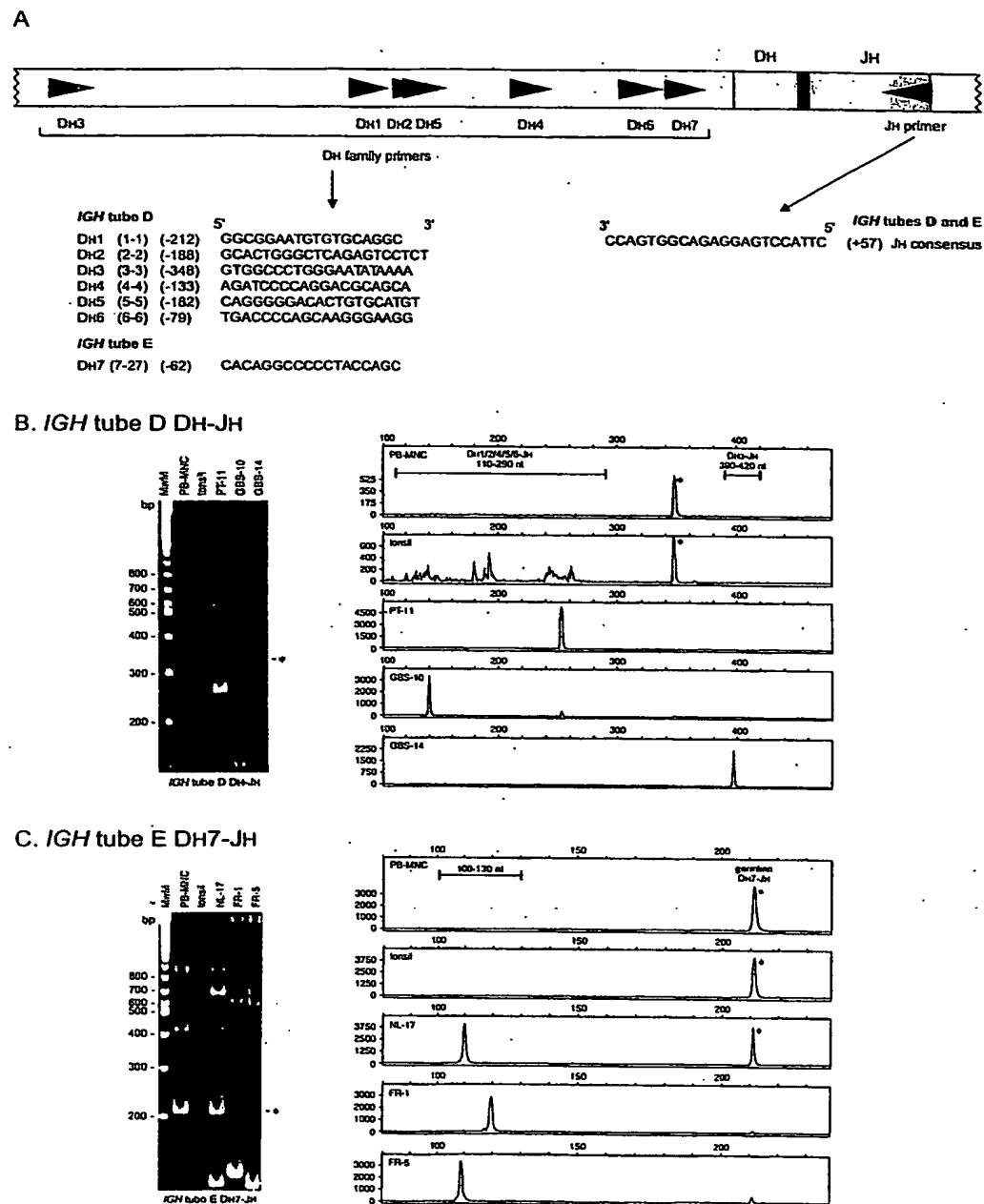
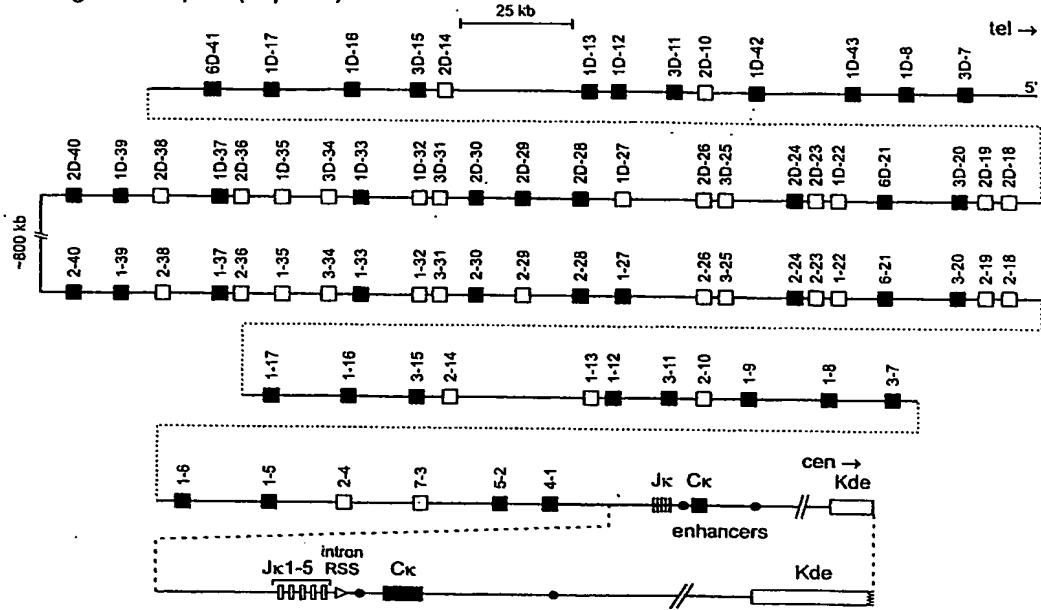
C. *IGH* tube A VH FR1-JHD. *IGH* tube B VH FR2-JHE. *IGH* tube C VH FR3-JH

Figure 3 (C, D and E)



A. *IGK* gene complex (#2p11.2)



B

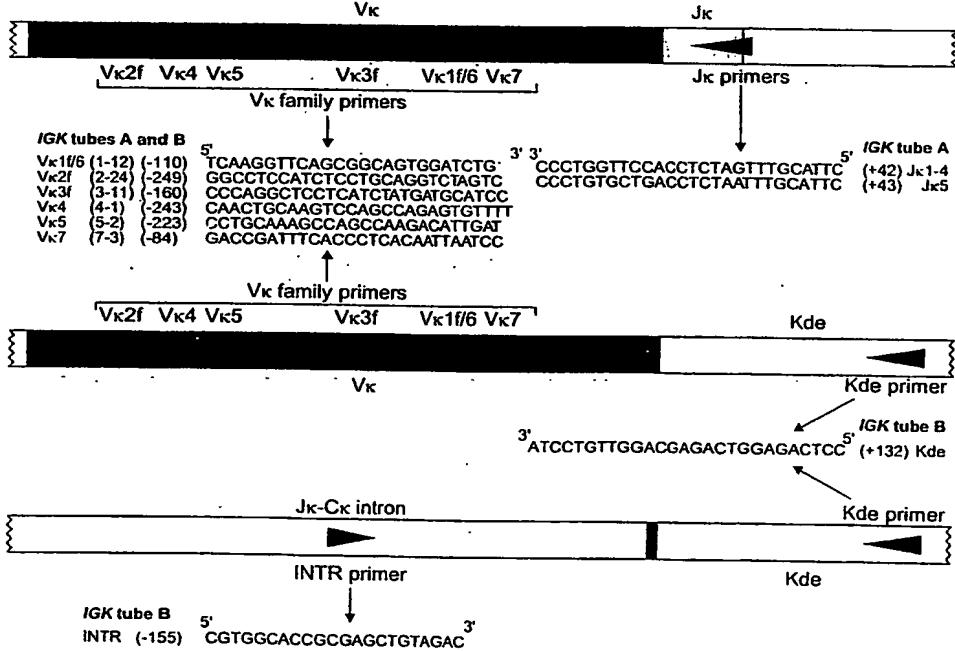
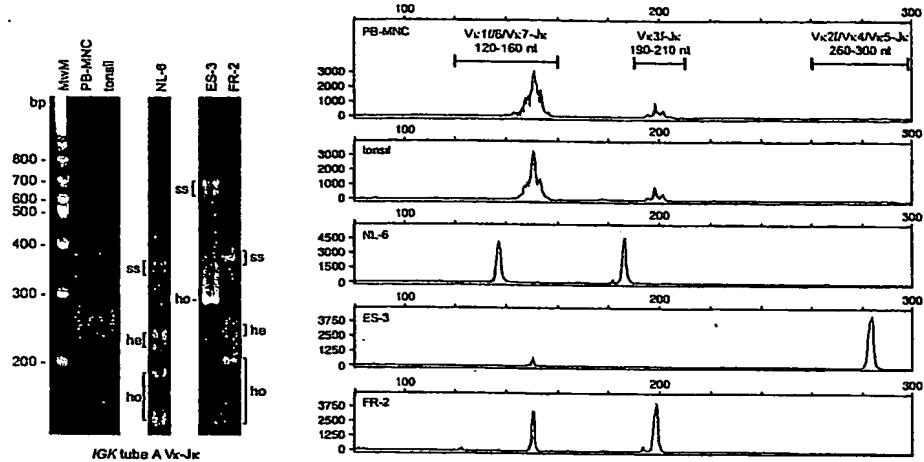


Figure 5 (A and B)

C. *IGK* tube A $V\kappa$ - $J\kappa$



D. *IGK* tube B $V\kappa$ /intron-Kde

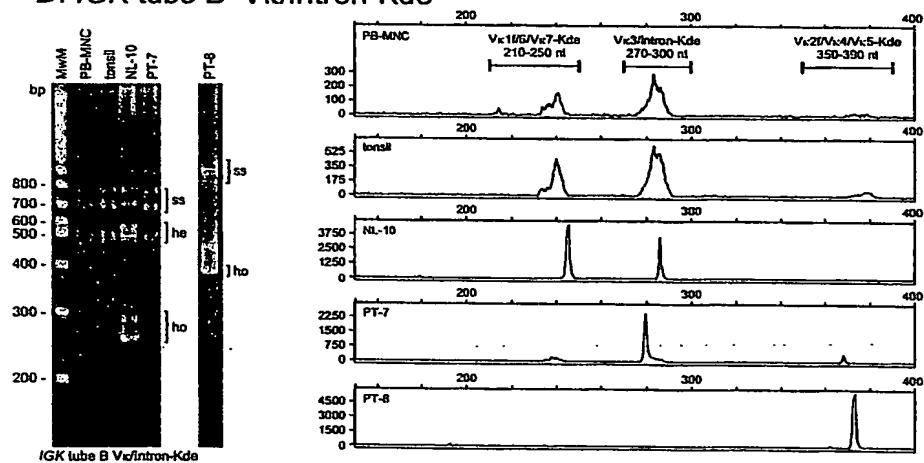
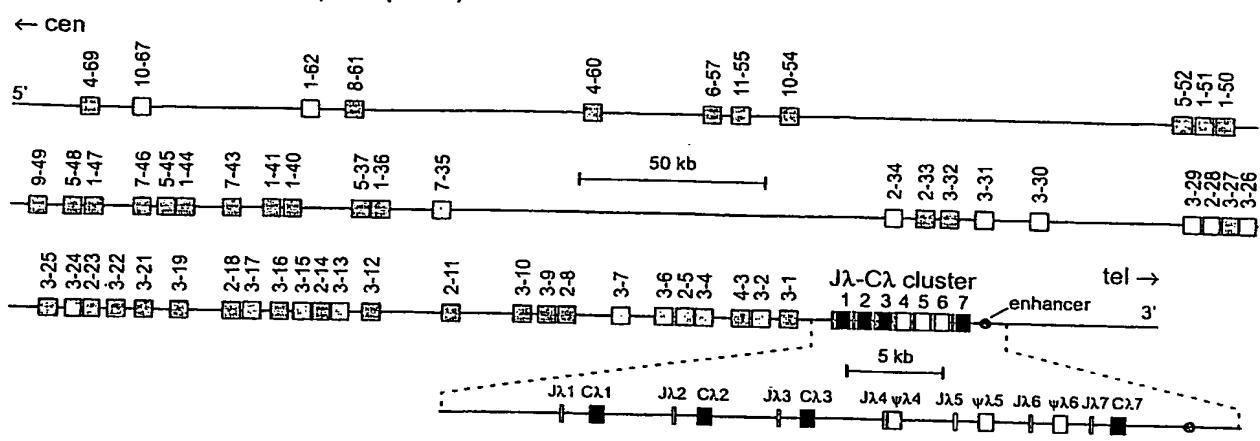


Figure 5 (C and D)

A. *IGL* gene complex (#22q11.2)

B

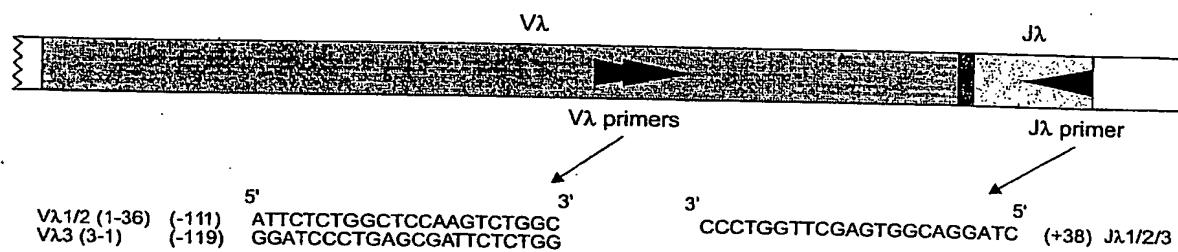
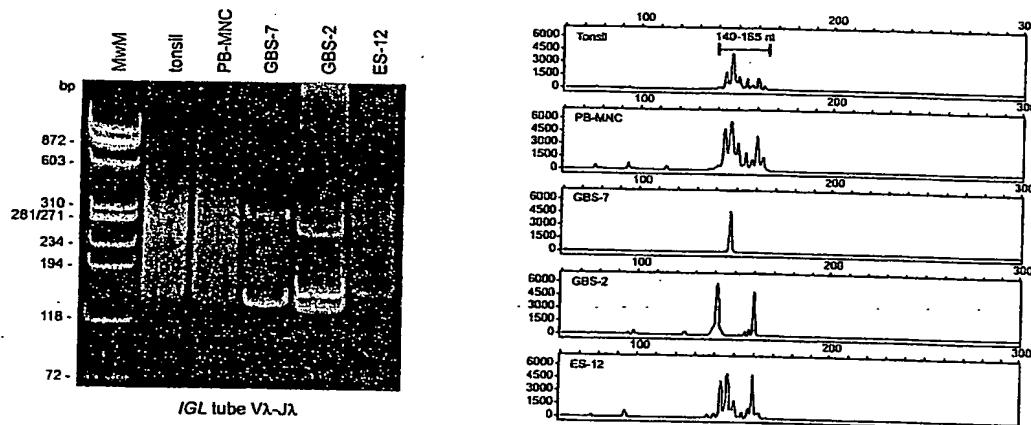
C. *IGL* tube *Vλ*-*Jλ*

Figure 6 (A, B and C)

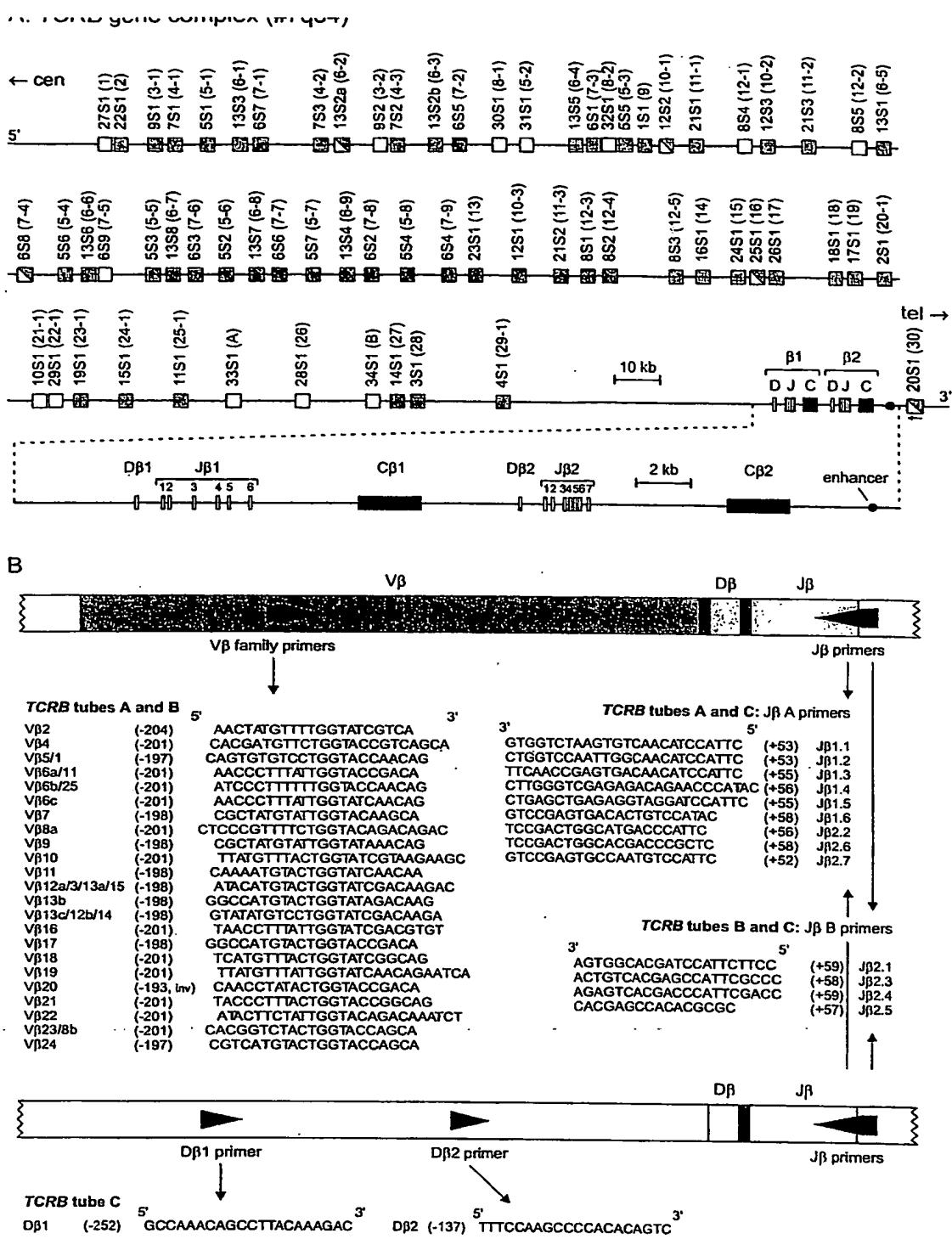
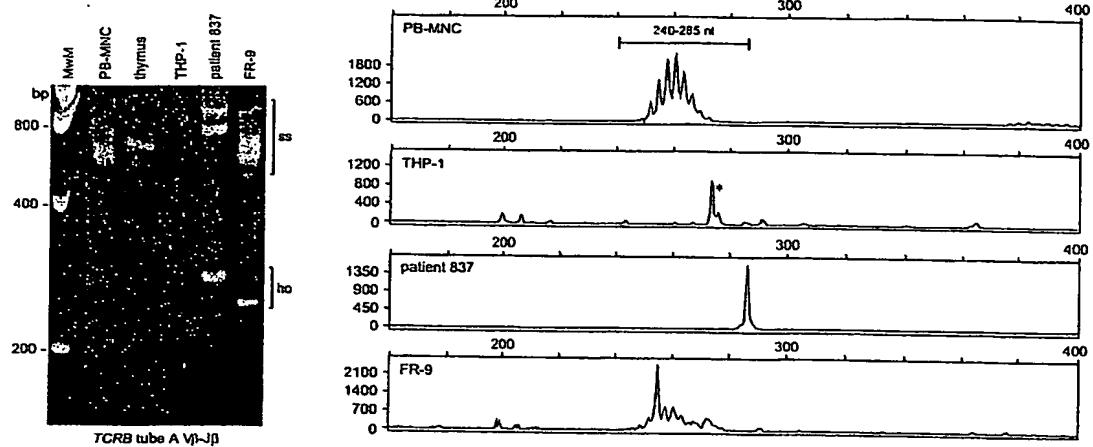
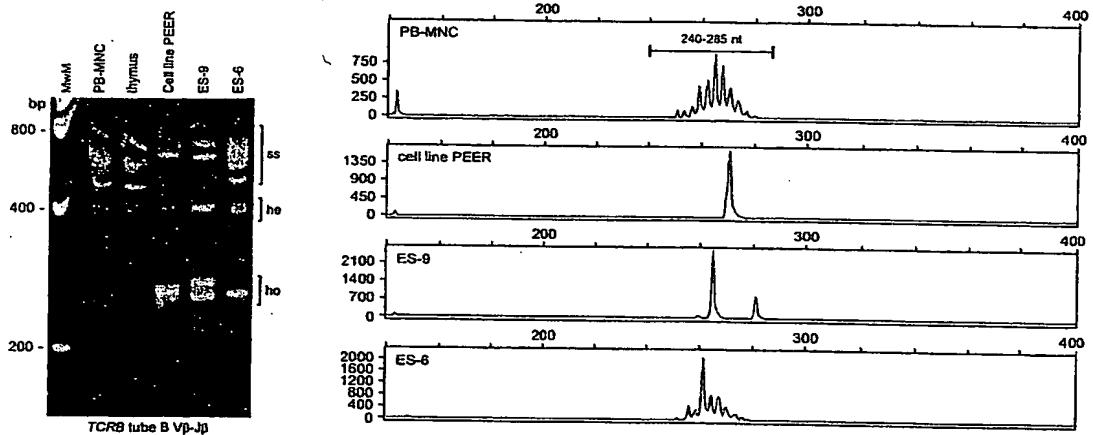
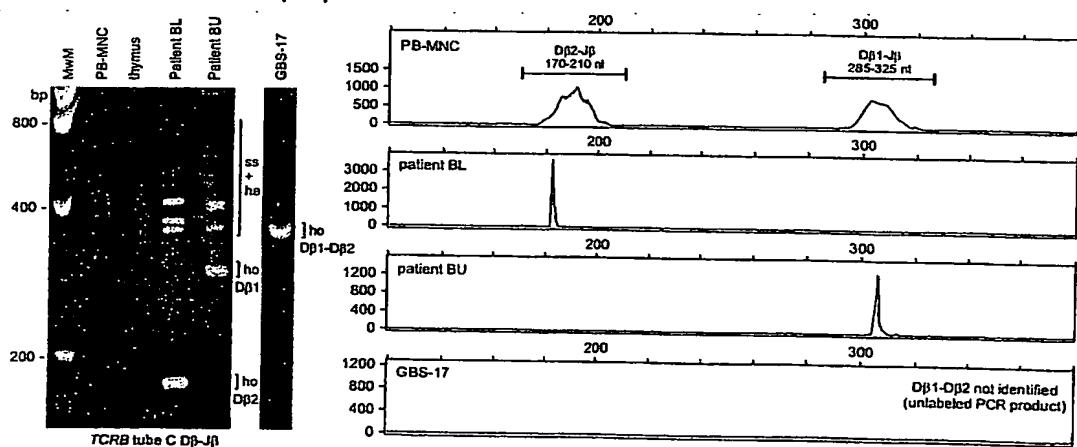
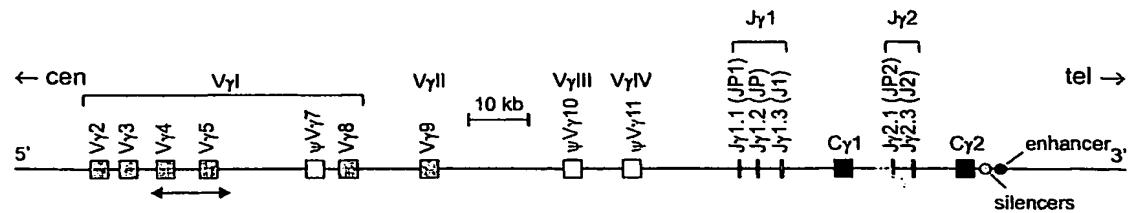


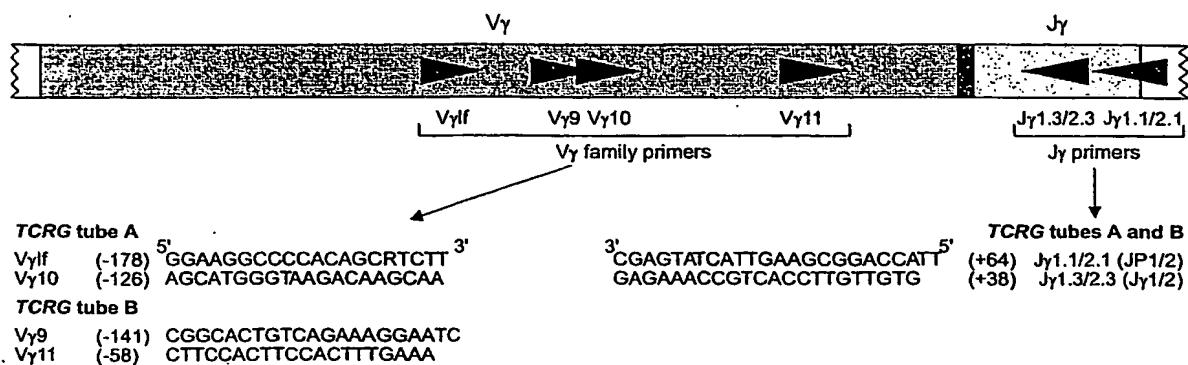
Figure 7 (A and B)

C. TCRB tube A V β -J β **D. TCRB tube B V β -J β** **E. TCRB tube C D β -J β** **Figure 7 (C, D and E)**

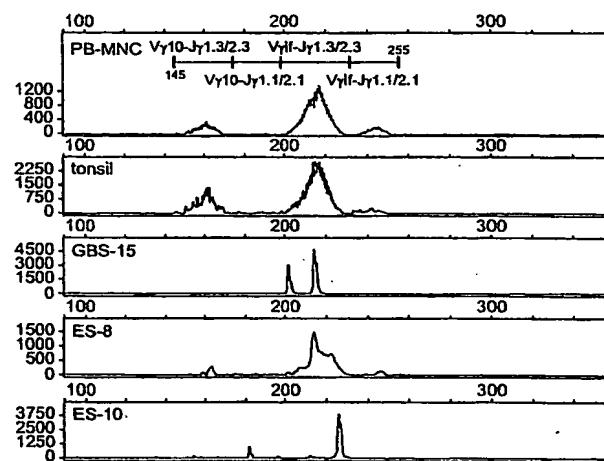
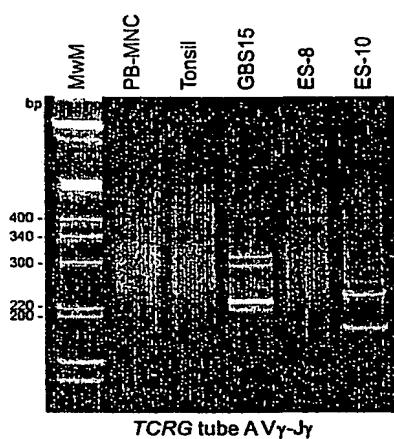
A. TCRG gene complex (#7p14)



B



C. TCRG tube A Vγ-Jγ



D. TCRG tube B Vγ-Jγ

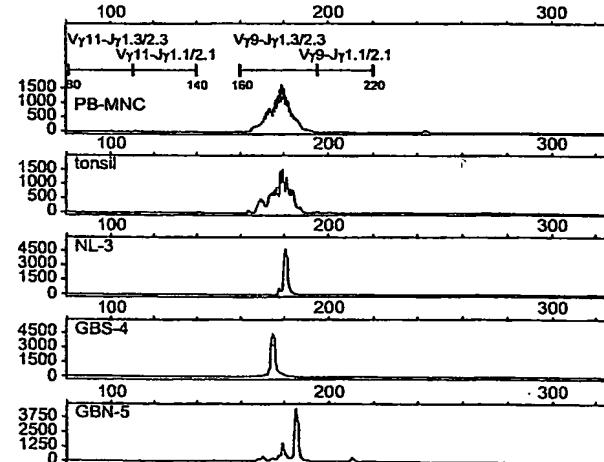
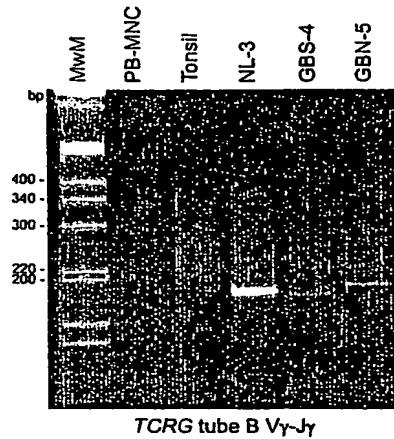
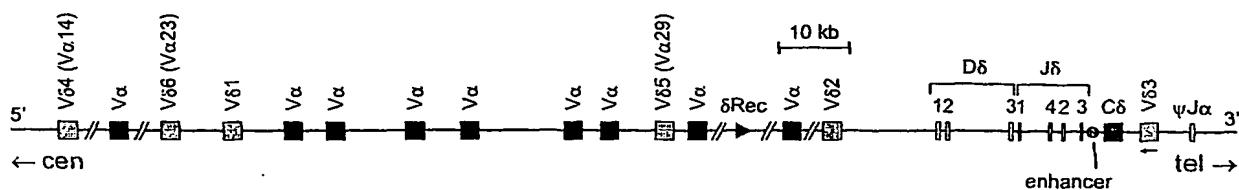
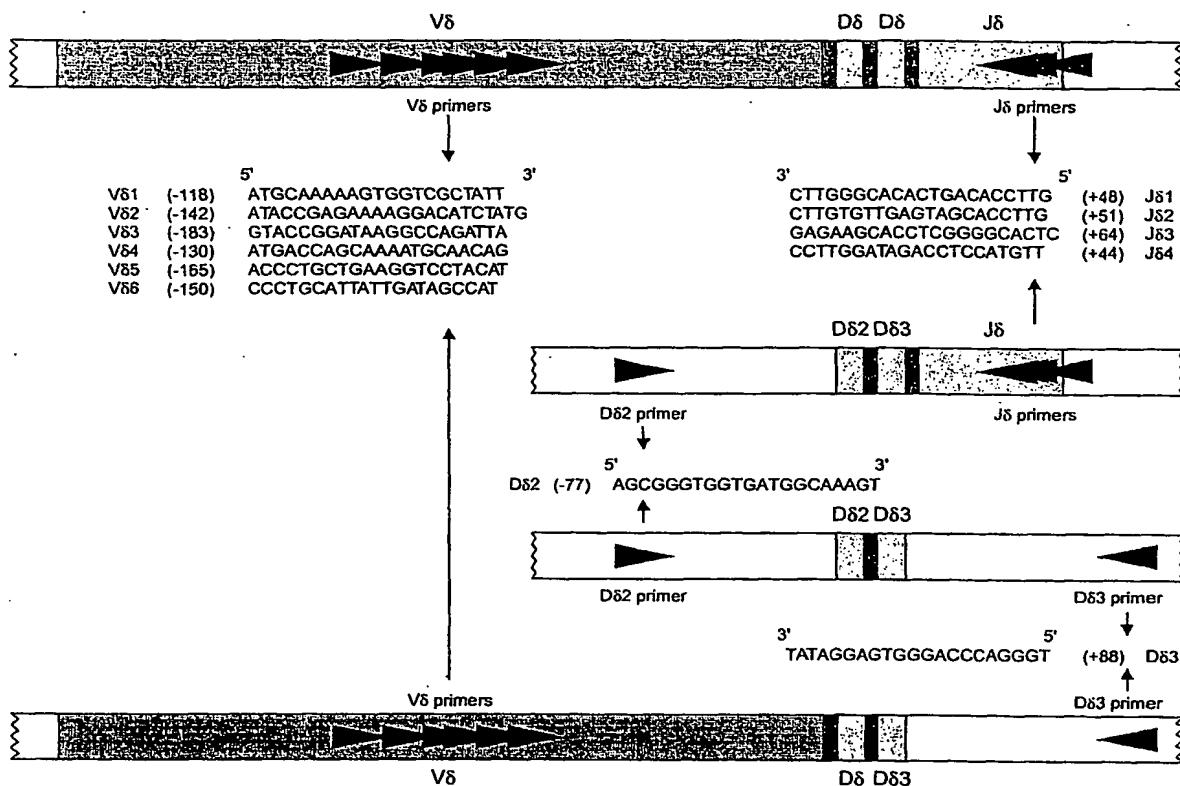


Figure 8 (A, B, C, and D)

A. TCRD gene complex (#14q11.2)



B



C. TCRD tube Vδ-Jδ/Dδ-Dδ/Dδ-Jδ

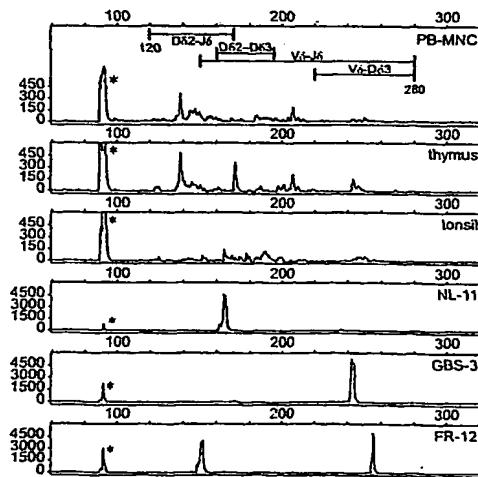
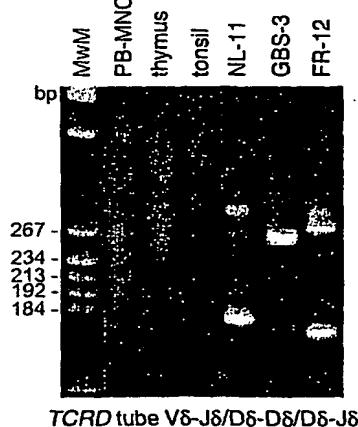
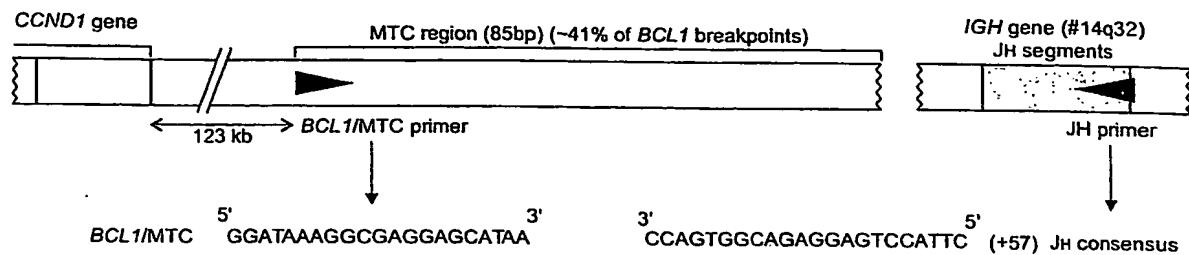


Figure 9 (A, B, and C)

A. *BCL1* locus (#11q13)



B. t(11;14) tube *BCL1* MTC-JH

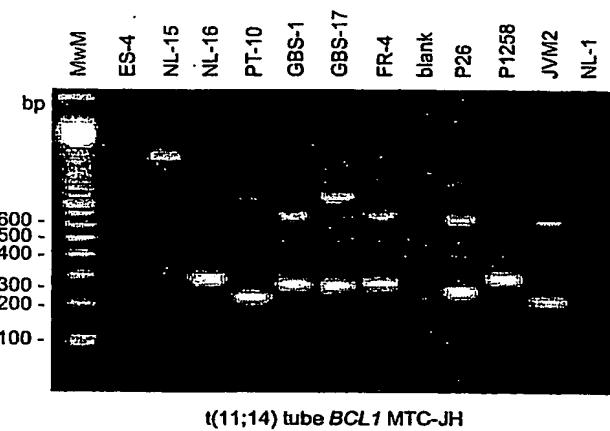
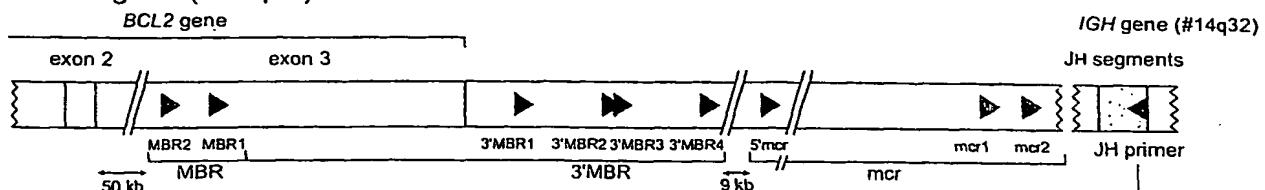


Figure 10 (A and B)

A. *BCL-2* gene (#18q21)



t(14;18) tube A: MBR primers 5' GACCA GAGATTCAAATCTATGG 3' 3' CCAGTGGCAGAGGAGTCCATTC 5' t(14;18) tubes A, B, and C
 MBR1 (3'end of exon 3) (-3072) MBR2 (3'end of exon 3) (-3575) ACTCTGTGGCATTAATGCATTATAT (+57) JH consensus

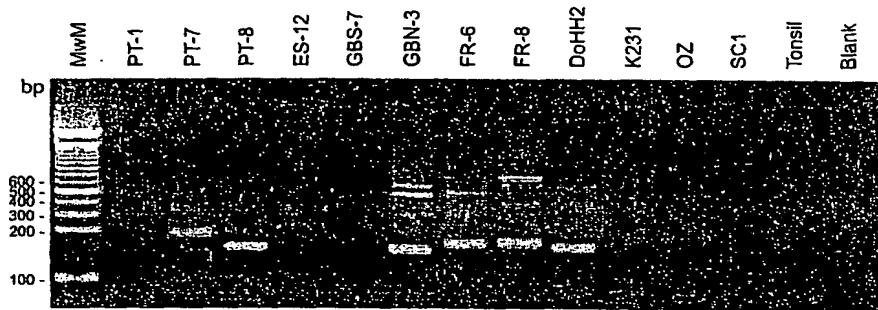
t(14;18) tube B: 3'MBR primers

3'MBR1 (3'end of exon 3) (+549)	GCACCTGCTGGATAACACACTG
3'MBR2 (3'end of exon 3) (+1224)	AAACTAGCAGGGTGTGGTGGC (replaced by +1362; GGTGACAGAGCAAAACATGAACA)
3'MBR3 (3'end of exon 3) (+1619)	TAATGACTGGGGAGCAAATCTT
3'MBR4 (3'end of exon 3) (+2550)	ACTGGTTGGCGTGGTTAGAGA

t(14;18) tube C: mcr primers

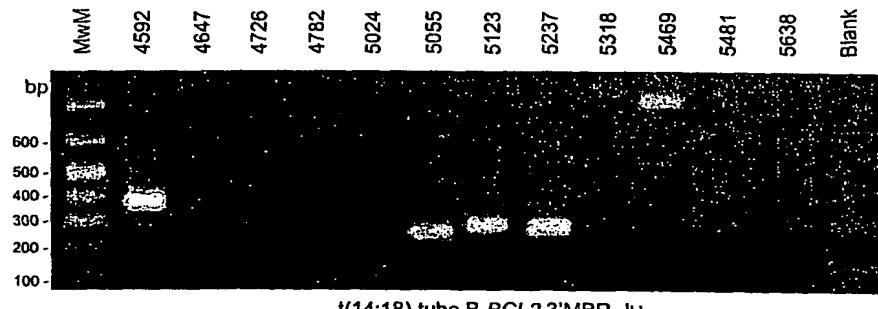
5'mcr	(3'end of exon 3)	(+15681)	CCTTCTGAAAGAACGAAAGCA
mcr1	(file AF275873)	(+1961)	TAGAGCAACGCCGCAATAATAA
mcr2	(file AF275873)	(+2407)	TGAATGCCCCTCTCAAATCAA

B. t(14;18) tube A BCL2 MBR-JH



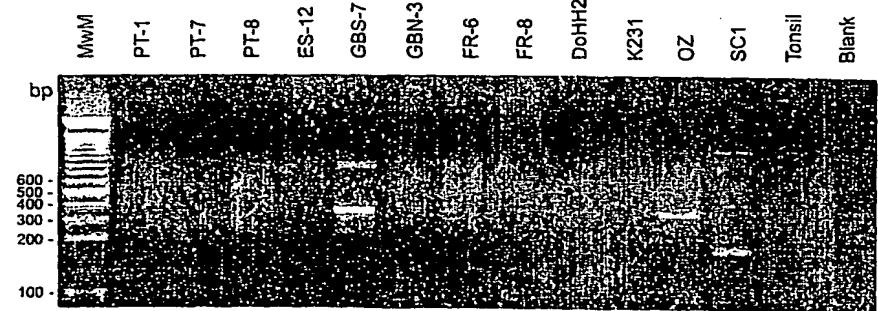
t(14;18) tube A *BCL2* MBR-JH

C. t(14;18) tube B *BCL2* 3'MBR-JH



t(14;18) tube B *BCL2* 3'MBR-JH

D. t(14;18) tube C *BCL2* mcr-JH



t(14;18) tube C *BCL2* mcr-JH

Figure 11 (A, B, C, and D)

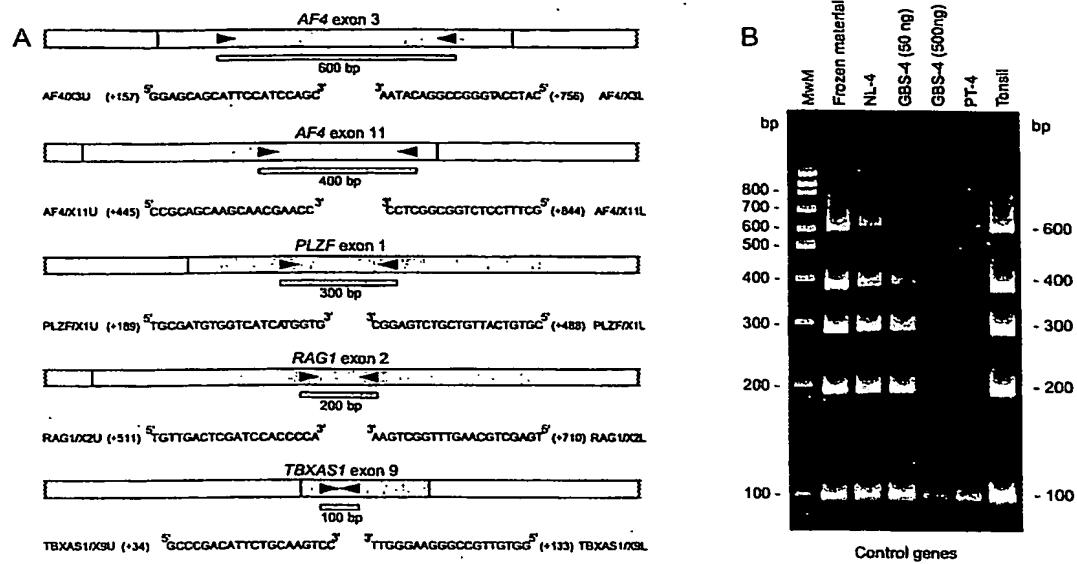


Figure 12 (A and B)

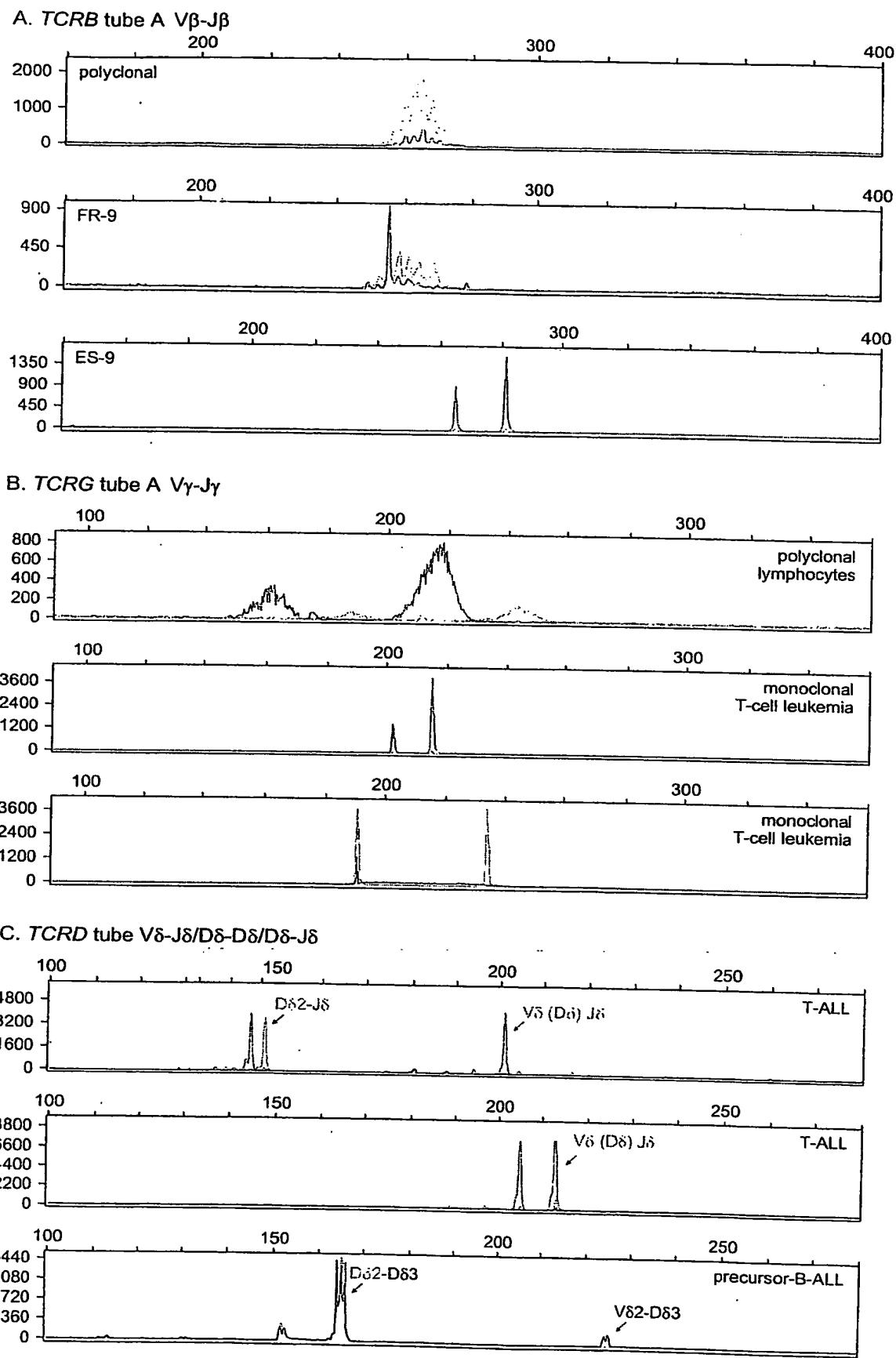


Figure 13 (A, B and C)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date
22 April 2004 (22.04.2004)

PCT

(10) International Publication Number
WO 2004/033728 A3

(51) International Patent Classification⁷: **C12Q 1/68**

(21) International Application Number:
PCT/NL2003/000690

(22) International Filing Date: 13 October 2003 (13.10.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/417,779 11 October 2002 (11.10.2002) US

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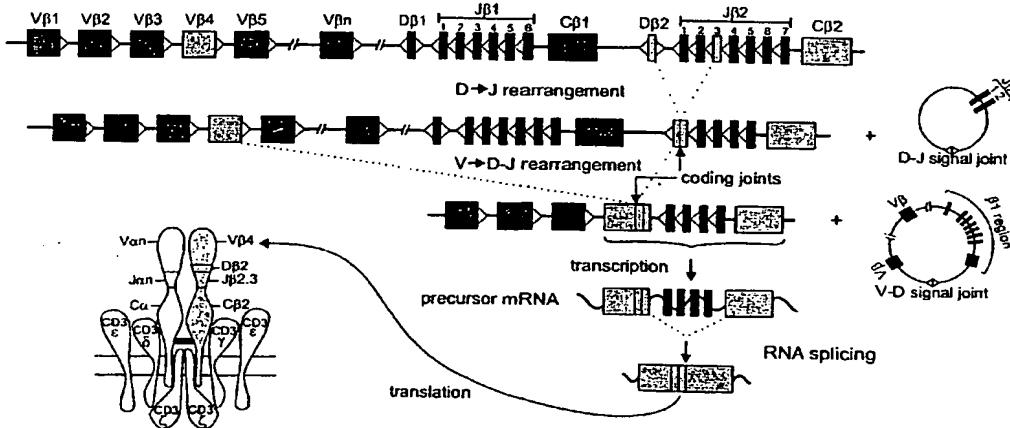
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[Continued on next page]

(54) Title: NUCLEIC ACID AMPLIFICATION PRIMERS FOR PCR-BASED CLONALITY STUDIES



(57) Abstract: The invention relates to PCR-based clonality studies for among others early diagnosis of lymphoproliferative disorders. Provided is a set of nucleic acid amplification primers comprising a forward primer, or a variant thereof, and a reverse primer, or a variant thereof, capable of amplifying a rearrangement selected from the group consisting of a VH-JH IGH rearrangement, a DH-JH IGH rearrangement, a VK-JK IGK rearrangement, a VK/intron-Kde IGK rearrangement, a V λ -J λ IGL rearrangement, a V δ -J δ TCRB rearrangement, a DB-JB TCRB rearrangement, a V γ -J γ TCRG rearrangement, a V δ -J δ TCRD rearrangement, a D δ -D δ TCRD rearrangement, a D δ -J δ TCRD rearrangement, a V δ -D δ TCRD rearrangement, or a translocation selected from t(11;14)(BCL1-IGH) and t(14;18)(BCL2-IGH). The primers can be used in PCR-based clonality studies for early diagnosis of lymphoproliferative disorders and detection of minimal residual disease (MRD). Also provided is a kit comprising at least one set of primers of the invention.

WO 2004/033728 A3

(81) **Designated States (national):** AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, EG, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,

ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) **Date of publication of the international search report:**

12 August 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No

/NL 03/00690

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/00848 A (BUSCH CHRISTER ;ASCENDIA AB (SE); LUNDIN TOMAS (SE); MAANSSON PER) 3 January 2002 (2002-01-03) example 1 -----	1,20,21, 37-42
Y	US 6 312 690 B1 (EDELMAN L EACUTE NA ET AL) 6 November 2001 (2001-11-06) column 7 -----	1,20,21, 37-42
Y	US 5 472 693 A (MEZES PETER S ET AL) 5 December 1995 (1995-12-05) SEQ ID NO.23 -----	1,20,21, 37-42

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- *A* document defining the general state of the art which is not considered to be of particular relevance
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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the International search

3 March 2004

Date of mailing of the international search report

16. 06. 2004

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Knudsen, H

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEANE M ET AL: "IMMUNOGLOBULIN GENE FINGERPRINTING AN APPROACH TO ANALYSIS OF B LYMPHOID CLONALITY IN LYMPHOPROLIFERATIVE DISORDERS" BRITISH JOURNAL OF HAEMATOLOGY, vol. 77, no. 3, 1991, pages 274-281, XP009026476 ISSN: 0007-1048 abstract; table 1	1,20,21, 37-42
Y	WO 00/22111 A (MEDICAL RES COUNCIL ;CUMBERS SARAH JANE (GB); NEUBERGER MICHAEL SA) 20 April 2000 (2000-04-20) example 1	1,20,21, 37-42
A	BARKER R L ET AL: "CYTOMETRIC DETECTION OF DNA AMPLIFIED WITH FLUORESCENT PRIMERS: APPLICATIONS TO ANALYSIS OF CLONAL BCL-2 AND IGH GENE REARRANGEMENTS IN MALIGNANT LYMPHOMAS" BLOOD, W.B. SAUNDERS, PHILADELPHIA, VA, US, vol. 83, no. 4, 15 February 1994 (1994-02-15), pages 1079-1085, XP002923853 ISSN: 0006-4971 the whole document	1,20,21, 37-42
A	WO 00/28086 A (IVS TECHNOLOGIES LLC ;MILLER JEFFREY E W (US)) 18 May 2000 (2000-05-18) page 22; figure 1B	1,20,21, 37-42

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 03/00690

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 20, 21, 37-42 (partially)

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1: claims 1,20,21,37-42 (partially)

A set of primers comprising primers binding to FR1 and one primer binding to JH (tube A in Fig.3B). Method and kit for use in amplification and detection of rearrangements.

Invention 2: claims 1,20,21,37-42 (partially)

A set of primers comprising a forward primer binding to FR2 and a reverse primer binding to JH (tube B in Fig 3B). Method and kit for use in amplification and detection of rearrangements.

Invention 3: claims 1,20,21,37-42 (partially)

A set of primers comprising a forward primer binding to FR3 and a reverse primer binding to JH (tube C in Fig 3B). Method and kit for use in amplification and detection of rearrangements.

Invention 4: claims 2,22 (completely), 20,37-42 (partially)

A set of primers wherein the forward primer is selected from DH primers and the reverse primer is the JH conservative primer shown in Fig 4A. Method and kit for use in amplification and detection of rearrangements.

Invention 5: claims 3-4,23-24 (completely), 20,37-42 (partially)

A set of primers wherein the forward primer is selected from V kappa or INTR primers and the reverse primer is a J kappa primer shown in Fig 5B. Method and kit for use in amplification and detection of rearrangements.

Invention 6: claims 5,25 (completely), 20,37-42 (partially)

A set of primers wherein the forward primer is selected from the V lambda primers shown in Fig.6B and the reverse primer is the J lambda primer shown in Fig.6B. Method and kit for use in amplification and detection of rearrangements.

Invention 7: claims 6,20,26,37-42 (partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A set of primers comprising a forward primer binding to V 2 and a reverse primer selected from the J A and J B primers shown in Fig 7B. Method and kit for use in amplification and detection of rearrangements.

Invention 8: claims 6,20,26,37-42 (partially)

A set of primers comprising a forward primer binding to V 4 and a reverse primer selected from the J A and J B primers shown in Fig 7B. Method and kit for use in amplification and detection of rearrangements.

Inventions 9-25: claims 6,20,26,37-42 (partially)

Each invention concerns a set of primers comprising a forward primer binding to one of Vbeta5 etc to Vbeta24 and a reverse primer selected from the JbetaA and JbetaB primers shown in Fig 7B. Method and kit for use in amplification and detection of rearrangements.

Invention 26: claims 7,27 (completely), 20,37-42 (partially)

A set of primers comprising a forward primer binding to Dbeta and a reverse primer selected from the JbetaA and JbetaB primers shown in Fig 7B. Method and kit for use in amplification and detection of rearrangements.

Invention 27: claims 8,28 (completely), 20,37-42 (partially)

A set of primers comprising a forward primer binding to Vgamma and a reverse primer selected from the Jgamma primers shown in Fig 8B. Method and kit for use in amplification and detection of rearrangements.

Invention 28: claims 9-12,29-32 (completely), 20,37-42 (partially)

A set of primers capable of amplifying TCRD rearrangements comprising a forward primer and a reverse primer binding to Vdelta, Ddelta or Jdelta shown in Fig 9B. Method and kit employing the primers for use in amplification and detection of rearrangements.

Invention 29: claims 13,33 (completely), 20,37-42 (partially)

A set of primers comprising a forward and a reverse primer binding to BCL1/MTC as shown in Fig.10A. Method and kit employing the primers for use in amplification and detection of rearrangements.

Invention 30: claims 14,34 (completely), 20,37-42 (partially)

A set of primers comprising a forward primer binding to MBR and a reverse primer binding to the JH consensus sequence as defined in Fig.11A. Method and kit employing the primers for use in amplification and detection of rearrangements.

Invention 31: claims 15 (completely), 35-36 (partially)

Sets of primers for amplification of human TBXAS1, RAG1, PLZF and exon3 and exon 11 of AF4 as defined in Fig.12A and method of using the primers for assessing the quality of a DNA sample.

Invention 32: claims 16 (completely), 35-36 (partially)

Sets of primers for amplification of human RAG1 as defined in Fig.12A and method of using the primers for assessing the quality of a DNA sample.

Invention 33: claims 17 (completely), 35-36 (partially)

Sets of primers for amplification of human PLZF as defined in Fig.12A and method of using the primers for assessing the quality of a DNA sample.

Invention 34: claims 18-19 (completely), 35-36 (partially)

Sets of primers for amplification of exon3 and exon 11 of human AF4 as defined in Fig.12A and method of using the primers for assessing the quality of a DNA sample.

INTERNATIONAL SEARCH REPORT

national Application No

T/NL 03/00690

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